Investigating the evolution of herbicide resistance in UK populations of *Alopecurus Myosuroides*

Craig Martin Knight

A thesis for the degree of Doctor of Philosophy

Submitted to the School of Life Sciences, University of Warwick

September 2015

Contents

List of Figures

List of Tables

Acknowledgements

First of all, I would like to thank my supervisor Dr. Paul Neve. Throughout the PhD, Paul has provided a level of guidance and feedback that has enabled me to fully understand and engage with the subject of herbicide resistance evolution, so that the research could be successfully completed to high scientific standard. I would also like to thank the BBSRC for financially supporting the project.

I would like to express appreciation to Bayer CropScience for supporting this research with their expertise, facilities and financial support. In particular I would like to thank Dr. Roland Beffa and Dr. Gordon Anderson-Taylor, for facilitating my visits to Bayer CropScience in Frankfurt, providing constant guidance that has benefitted quality of the research beyond measure, and for supporting the research in the UK. I owe a large debt of gratitude to Ranghild Paul, Thomas Schubel, Johannes Hermann, Rebecca Drücker, Stephanie Bagdahn, Falco Peter, and Ben Coombs for their help throughout the project, and for making me feel welcome during my time working in Frankfurt.

I would also like to thank all past and present members of the weed research group at the University of Warwick; in particular Laura Davies for her help over the part four-years. I also acknowledge the help provided by the University of Warwick's horticultural services team in the set up and running of glasshouse experiments, Andrew Mead for his help with statistical analysis, and Prof. Richard Napier for his supervision.

Finally, I would like to thank my family for all of their support and encouragement over the past four years; especially my wife Bethan, parents Sharon and Paul, brothers Glen and Aaron, and last, but by no means least, Trillian.

Declaration

This thesis is presented in accordance with the regulations for the degree of doctor of philosophy. It has been composed by myself and has not been submitted in any previous application for any degree. The author has undertaken the work in this thesis.

Summary

Alopecurus myosuroides, a problematic weed of UK winter cereals, is predominantly controlled by post-emergent herbicides with ALS and ACCase modes of action (MOA). Evolved resistance to these MOA - endowed by the mechanisms of target-site (TSR) and/or enhanced metabolism (EMR) – threatens the sustainable production of winter cereals in the UK. This project aims to establish the frequency of ALS and ACCase resistance in UK populations of *A. myosuroides* and the factors that drive its evolution. From a 2011 survey of 92 UK *A. myosuroides* populations, mesosulfuron-methyl + iodosulfuron-methyl-sodium (ALS) resistance was confirmed in 81 populations; all 92 populations exhibited clodinofop-propargyl (ACCase) resistance. To understand how management affects resistance evolution, seventeen populations from the 2011 survey were resampled (2012–2014) so that estimated frequencies of phenotypic resistance, TSR and EMR could be compared to weed management histories. Fields in which spring crops were more frequently planted possessed *A. myosuroides* that exhibited lower levels of phenotypic resistance to both ALS and ACCase MOA. A simulation model was developed to describe *A. myosuroides* herbicide resistance evolution. However, this model could not be validated when parameterized with resistance and management data collected from the UK. In the 2011 survey, homozygous Pro-197-Thr ALS TSR mutations were absent. Phenotyping, germination, and genotyping experiments of plants from controlled heterozygous Pro-197-Thr crosses confirmed that there is a lethality associated with homozygous Pro-197-Thr mutations. To test the hypothesis that pre-existing ACCase EMR increases the rate of ALS EMR selection, four *A. myosuroides* populations – three with ACCase EMR and one without - were selected over two generations with ALS herbicide. From dose-response analyses of survival, the three populations with ACCase EMR exhibited significant increases in ED_{50} values after selection, whereas the population without ACCase EMR did not.

1.0 Literature Review

1.1 Agricultural Weeds – A Global Issue

1.1.1 The importance of agriculture in the 21st century

Estimates of human population size currently stand at 7.13 billion (United Nations 2004), with approximately one in eight people, largely from developing regions, suffering from chronic hunger (FAO 2013a). It is predicted that the global population will increase annually by 0.8% (on average) to 9.6 billion in 2050 (FAO 2009; United Nations 2013). This rise in population, coupled with a growth in the gross domestic product (GDP) of both developing and developed countries, is expected to exacerbate demand for food to a level 70% higher than what it is now (FAO 2009). This makes food security: ensuring that food is sustainably produced, correctly utilized, and accessible to those who need it (FAO 2013a) important to maintaining and improving the lives of millions of people worldwide, now and in the future.

The ever-increasing quantities of sustainably produced food required for global population subsistence remain unrealised, and will continue to do so if numerous agronomic challenges are not met head on. Climate change, biodiversity loss, land-use change, pests and disease, all have the potential to decrease agricultural outputs (FAO 2013a). To ensure global food security, the use of agricultural sciences to promote the distribution, preservation, use, and sustainable intensification of arable yields is essential (UK Government 2013). One area of agricultural science integral to yield maintenance is the control of pests (pathogens, virus', animals and plants); if left unmanaged, infestations can arise that significantly limit the productive potential of an arable system (Oerke 2006).

1.1.2 Arable weeds

At present, the largest pest related losses in global agricultural production are attributed to pathogens and animal pests. However plants, or more specifically weeds, have the potential to negatively affect yield, even more so than pathogens and animal pests if there is a failure to control them (Oerke 2006) (Figure 1.1).

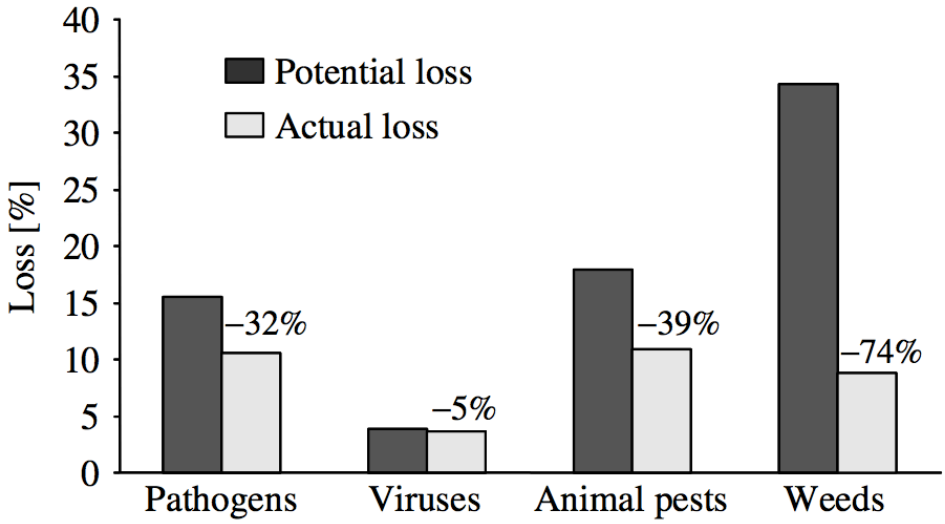


Figure 1.1: Pest control practices and their average efficacies in reducing crop losses to pathogens, viruses, animal pests, and weeds globally. The light grey bars indicate actual losses, while the dark grey bars indicate the estimated potential loss if there is a failure to control the pest in question (Source: Oerke 2006).

There have been many definitions of a weed: 'a plant not valued for its use or beauty', 'a plant whose virtues have yet to be discovered' (Naylor & Drummond 2002); a generic definition that could be considered representative of all of these is 'a plant growing where it is not wanted and in competition with cultivated plants' (OED 2015). The definition used here is 'a plant that has evolved ecological characteristics that adapt it to survive and compete on highly disturbed agricultural land, with the potential to reduce the quality and/or yield of a crop when environmental conditions are favourable' (Naylor & Drummond 2002). The

ecological characteristics of an 'ideal weed' (a purely abstract concept) have been listed by Baker (1974) (Table 1.1); if a plant lacks these characteristics, they will not manifest as a weed (Baker 1974). Characteristics such as these enable weeds to thrive in an agricultural environment; however, if this land ceases to be disrupted by cultivation, weeds will be overcome as they struggle to compete with the non-weed plant communities that will become established (Baker 1974).

Table 1.1: The twelve characteristics of an 'ideal weed', as described by Baker (1974). If a plant possess' a large number of these characteristics then it is likely to be considered a weed (Source: Baker 1974).

	Characteristics of an 'Ideal Weed'
1.	Germination requirements fulfilled in many environments
2.	Discontinuous germination (internally controlled) and great longevity of seed
3.	Rapid growth through vegetative phase
4.	Continuous seed production for as long as growing conditions permit
5.	Self compatible but not completely autogamous or apomictic
6.	When cross-pollinated, unspecialised visitors or wind utilized
7.	Very high seed output in favourable environmental circumstances
8.	Produces seed in wide range of environmental conditions; tolerant and plastic
9.	Has adaptations for short- and long-distance dispersal
10.	If perennial, has vigorous vegetative reproduction / regeneration from fragments
11.	If a perennial, has brittleness, so not easily drawn from ground
12	Has ability to compete inter-specifically by special means (rosette, choking growth, allelochemicals)

1.1.3 Weeds: a problematic agricultural pest

Annual pest related yield loss has been estimated for six crops worldwide: *Triticum aestivum* (wheat) *Oryza sativa* (rice), *Zea mays* (maize), *Solanum tuberosum* (potatoes), *Glycine max* (soybean), and *Gossypium hirsutum* (cotton) (Table 1.2, Oerke 2006). Although weeds are not the principle source of pest related yield loss in most crops, they contribute largely to each crops total annual yield loss (Table 1.2). Estimates of the financial impact of weed related yield losses are few and those that

do exist may be out dated. For example in the USA, weeds have been estimated to cause $4.1 billion worth of crop loss each year (Bridges and Anderson 1992), while in Australia weeds have been estimated to cause an annual yield loss of $3.9 billion (Sinden *et al* 2004).

Table 1.2: Actual annual crop losses due to pests globally. Estimated yield loss for six crops as a result of the four main groups of pest (weeds, animal pests, pathogens and viruses), and total yield loss for all pests combined (Source: Oerke 2006).

	Percentage (%) crop loss due to				
Crop	**Weeds**	**Animal Pests**	**Pathogens**	**Viruses**	**Total**
Wheat	7.7	7.9	10.2	2.4	28.2
Rice	10.2	15.1	13.5	1.4	37.4
Maize	10.5	9.6	8.5	2.7	31.2
Potatoes	8.3	10.9	14.5	6.6	40.3
Soybean	7.5	8.8	8.9	1.2	26.3
Cotton	8.6	12.3	7.2	0.7	28.8

Competition for light, nutrients, water and space between weed and crop, is the principle reason why weeds can be so damaging to yields; additionally, weeds have the potential to host and facilitate the spread of other generalist pests and diseases to the crop (Naylor and Drummond 2002). It is for these reasons that, for as long as there have been arable weeds, farmers and agronomists have endeavoured to eradicate them from their crops to maximize yield.

1.2 Agricultural Weed Management

1.2.1 'Classical' weed management practices

Agriculture has been practiced for at least the past 10,000 years (Campbell and Reece 2005). Since the advent of cultivation, farmers have been battling to control weed species that have invaded their crops to maintain yield. For centuries, animal

drawn ploughs and hand/hoe weeding by farm workers were the only methods for removing weeds from a crop (Naylor and Drummond 2002). As of the mid-18th century and the advent of the industrial revolution, steam and combustion engine powered mechanical weeding advanced the capacity of farmers to control weeds over shorter time scales and larger areas. An increase in agricultural/ horticultural knowledge around this time also facilitated a greater appreciation of crop rotations, soil nutrient, irrigation, and pH levels as a means of weed management. For the past seventy years, the predominant method of arable weed control across the developed world has been the application of synthetic chemicals: herbicides (Naylor and Drummond 2002).

1.2.2 Chemical (herbicidal) weed management

1.2.2.1 Herbicide site of action

Herbicides are man-made synthetic compounds (that can be closely related to natural compounds e.g. glufosinate) designed to inhibit a specific site of action (SOA = molecule/enzyme/structure) within a plant (Heap 2014). The SOA forms part of a molecular pathway that, once inhibited, prevents the synthesis of essential product(s) downstream of the SOA: consequently, a deficiency in the pathways end product(s) results in plant death (Powles and Yu 2010). The first commercially available synthetic herbicide came to market in 1946 (Heap 1997): 2, 4-dichlorophenoxyacetic acid (2,4-D), a synthetic auxin herbicide highly active against dicotyledonous (broadleaved) weeds (Kearney and Kaufman 1975). 2, 4-D revolutionized the control of broadleaf weeds in pastures, lawns and cereal crops globally, increasing system productivity while reducing the economic inputs attributed to weed control (Mithila

et al 2011). Since the commercialization of 2,4-D, more sites of action have been discovered, resulting in the number of commercially available herbicides increasing.

1.2.2.2 Herbicide mode of action

A herbicides mode of action (MOA) is different from its SOA. The SOA is a molecule/enzyme/structure within a plant that the herbicide targets for inhibition to induce plant death. The MOA – consisting of the biochemical MOA and the biological MOA - describes all aspects of a herbicide's activity. The biochemical MOA describes the interaction between the herbicide and a specific SOA to induce plant death, while the biological MOA describes the timing and manner of herbicide application, and the way in which the herbicide is absorbed and translocated throughout the plant (Powles and Yu 2010). So far, 25 biochemical MOA are known for commercial herbicides (for several herbicides the biochemical MOA is not known), allowing for the control of a broad spectrum of weed species (Heap 2014).

MOA can be grouped by biochemical MOA (of which there are 25, as discussed above), by selectivity, by use, or by activity. When grouped by selectivity, herbicides can be either selective or non-selective, and broad spectrum or specific. Selective MOA only target arable weeds (and have no or negligible effect on the crop), while non-selective MOA (e.g. glyphosate or glufosinate) affect weeds and the crop alike. Broad-spectrum herbicides do not differentiate between monocotyledonous and dicotyledonous species, being highly efficacious against a wide range of weed taxa, whereas specific herbicides target either monocots or dicots (Peterson 2010). When grouped by use, a herbicide's MOA can fall into one of two categories: pre-emergent herbicides or post-emergent herbicides. Pre-emergent herbicides are applied to the

soil before crop emergence, killing seedlings and juvenile plants as they germinate and emerge. Post-emergent herbicides are applied after crop emergence, and are most effective against growing weeds up to a specific growth stage. When grouped by activity, an herbicide's MOA can be described as being either contact or systemic. Contact herbicides only inhibit the SOA of plant tissue that they come into contact with, while systemic herbicides have the ability to be absorbed and translocated to most tissues throughout the plant, and in particular to meristems (Peterson 2010).

1.2.3 The importance of chemical weed management in 21st century agriculture

Herbicides are now a critical part of weed management strategies applied to agricultural land across the world: a good example of this can be found in the UK. It was estimated that approximately 30% of the UK's 6.3 million hectares of arable land was treated with herbicides in 2012, translating to approximately 6500 tonnes of herbicide being applied (DEFRA 2013; FAO 2013b). Due to their high efficacy and low economic costs in comparison to classical cultural techniques, herbicides have become increasingly relied upon, often as the sole method of arable weed control across the world. In northern Europe, the high efficacy of herbicides has facilitated an increase in practices that promote the continuous monoculture of important crops (e.g. *T. aestivum* (wheat) within a minimal tillage system (Lutman *et al* 2013)). Herbicide management practices, such as these, are currently the main method used to control the most problematic weed of winter cereals in northern Europe: *Alopecurus myosuroides*.

1.3 *Alopecurus myosuroides* (Huds.)

1.3.1 Morphology

Colloquially known as slender foxtail, black twitch, and black-grass (Hubbard 1984; Fitter *et al* 1992), *Alopecurus myosuroides* is a member of the Poaceae (Gramineae or true-grasses) most closely related to *Alopecurus pratensis* (meadow foxtail) and *Alopecurus geniculatus* (water foxtail) (Grime *et al* 1988). A shallow rooted plant that can grow up to 80cm in height by means of erect tuft forming culms; individuals normally consist of multiple culms that are smooth, slender and round in appearance (Naylor 1972) (Figure 1.2a). The major features from which *A. myosuroides* can be identified include blunt membranous ligules (2-5mm), flat glabrous pointed green leaves (blades: 3-16cm long; 2-8mm wide), and sheaths that are basally purple and inflated at the tip (Figure 1.2c) (Hubbard 1984). The panicles (seed heads) of *A. myosuroides* are 1-12cm in length and 3-6mm wide, narrow and cylindrical throughout but tapering to a point (resembling a spike) (Figure 1.2b). Each panicle can produce up to 270 spikelets (seeds) (Figure 1.2d); each spikelet is single flowered, sometimes brown or purplish in colour, consisting of a caryopsis complete with lemma and glumes is and approximately 4-7mm in length (Naylor 1972).

1.3.2 Biology, physiology and phenology

The annual life cycle of *A. myosuroides* can be described from the period just after the production of seed in late summer, until the end of seed production the following year. The majority of *A. myosuroides* seed germination occurs within the top 5cm of the soil, as seed reserves are too small to allow seedlings to grow to the soil surface and emerge below this depth (Moss 2013). The seed has an innate dormancy that prevents germination immediately after production between June and August (Swain

et al 2006), meaning that eighty-percent of *A. myosuroides* seed then germinates in the autumn (September – November), with a second smaller flush of germination in the spring (March – May) (Moss 2013).

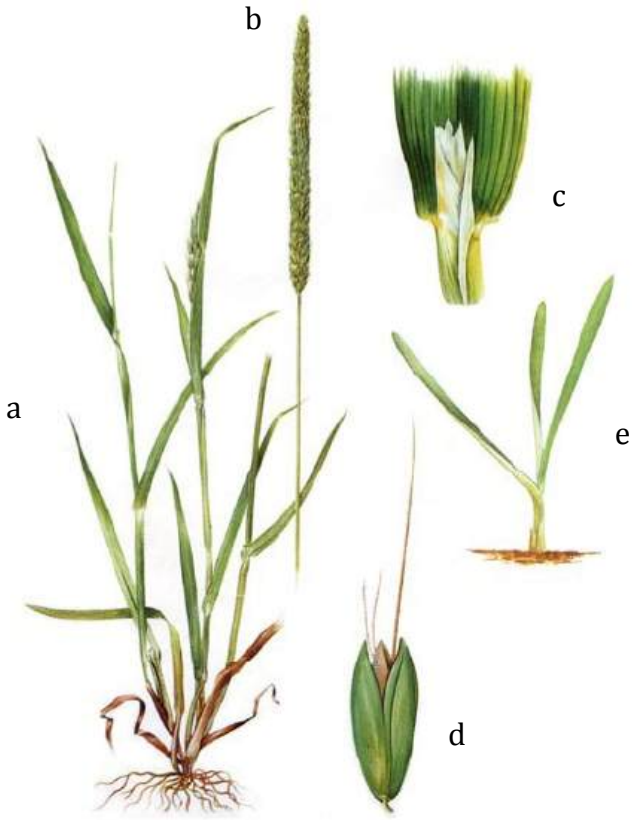


Figure 1.2: ***Alopecurus myosuroides***. A diagram showing some of the key morphological features of *A. myosuroides* (a) an *A. myosuroides* plant, showing its culms and flat leaves, (b) a panicle (seed head), (c) blunt membranous ligule, (d) germinating seed of *A. myosuroides*, (e) three-leaf stage of development. (Source: Geissel 2004).

Plants grow to maturity (morphologically described in section 1.3.1) until flowering, with reproduction via wind-pollinated allogamy taking place between May and August (Moss 2013). Seed is produced on seed heads, with fertilized seeds developing to maturity between June and August: Drought can have a detrimental effect on the production and maturation of seed (Naylor 1972).

Within a competitive wheat crop, one *A. myosuroides* plant can produce approximately twenty seed heads (Moss 2013); however, individuals with 150 head/plant have been recorded in situations with limited competition (Phillipson 1974). Each panicle is capable of producing around 100 seeds (Moss 2013); it has shown that seed shed in early June and late August has a lower viability than that of seed shed during late July, when seed shedding is at its peak (Moss 1983). With some *A. myosuroides* populations capable of producing patches in the field that have up to 500 heads/m^2, 50,000/m^2 seeds can be returned each year (Moss 2013). Seed survival within the soil diminishes annually by 73 – 83%, so after a period of three-years seed viability reduces to a level of between 1 – 3% (Moss 1985).

1.3.3 Occurrence

A temperate species, thought to be a native to Northern Europe (although absent from certain regions including Finland, Greenland and Faroe Islands), the Mediterranean, and extending to temperate Western Asia (Naylor 1972); an increase in global commerce over the past 200-years has also meant there have been numerous anthropogenic introductions of *A. myosuroides* to other temperate regions, e.g. China and North America (Holm 1991).

In the UK, France, Belgium, the Netherlands, and Germany, *A. myosuroides* is a predominant inhabitant of agricultural land (and to a lesser extent waste ground) (Figure 1.3). Since Figure 1.3 was published in 2010, *A. myosuroides* has become more prominent in Scotland, Ireland, Denmark, Sweden and Poland. The occurrence of *A. myosuroides* within an arable field depends upon the fields soil type and the

crop sown (Moss 2013), with *A. myosuroides* favouring water retentive clay or silt soils and autumn-sown crops, e.g. winter wheat (*T. aestivum*) or barley (*H. vulgare*) (Lutman *et al* 2013). *A. myosuroides'* dominance as an agricultural weed of winter cereal crops results from a combination of its competitive ability (within winter cereals) and high seed return. In recent years, the distribution of *A. myosuroides* has been expanding, spreading further north and east of its current distribution as a result of an increase in the planting of winter cereals, and climate change altering ambient conditions (notably temperature) to levels optimal for *A. myosuroides* (Holm 1991).

Figure 1.3: European distribution of *A. myosuroides* in northern Europe. The northern most limits of *A. myosuroides* distribution are shown by the solid black line: The main areas of northern Europe where *A. myosuroides* infestations occur are shown in the shaded areas (infestations outside of these regions are anecdotal). (Source: Délye *et al* 2010)).

Within the UK, *A. myosuroides* is a major weed of winter cereal crops. It is predominantly found in the southeast of the UK where arable cultivation is greatest and climatic conditions (i.e. summer temperatures above 15^{o}C) are favourable (Figure 1.4) (Hubbard 1984, Fitter *et al* 1992). Highly profitable winter cereals dominate the UK arable landscape, constituting 55% of the UK's cropping area in 2012 (DEFRA 2013). The effects of *A. myosuroides* on winter cereal production can be devastating; densities as low as 8 - 12 plants/m^2 can reduce yields by 2 – 5 % (Cussans 1991; Bayer Crop Science 2012), with 12 – 25 plants/m^2 resulting in losses of 0.4 – 0.8 t/ha (Moss 2013).

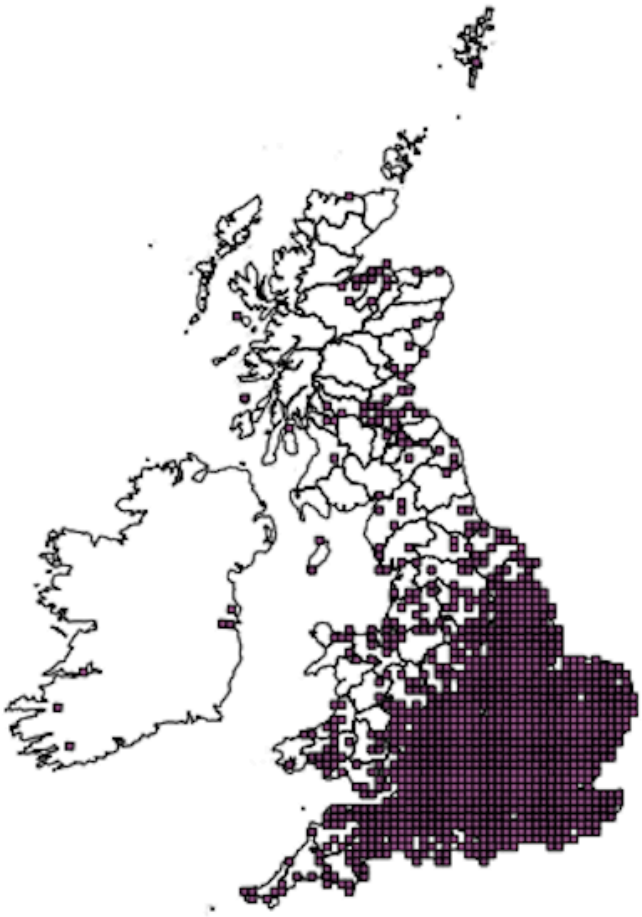


Figure 1.4: Distribution of *A. myosuroides* in the UK. The purple squares indicate where within the UK *A. myosuroides* has been identified. (Source: Online Atlas of British and Irish Flora (2013)).

Infestations of *A. myosuroides* that have escalated to densities of 100 plants/m^2 or even 300 plants/m^2, are capable of reducing yield by 1 – 2 t/ha, and 37% of total yield respectively (Roebuck 1987; Bayer Crop Science 2012). Continuous cereal cropping has exacerbated the presence and size of *A. myosuroides* populations in the UK, as shown by the number of farms applying herbicide (53% of 1350 surveyed) to specifically control *A. myosuroides* in 2011 (Bayer Crop Science 2012). Over the past 40 years, management of *A. myosuroides* has been over-reliant on application of herbicides, which has led to the evolution of herbicide resistant populations of *A. myosuroides*.

1.4 Herbicide resistance

1.4.1 Definition and brief history

A herbicide resistant plant can be defined as 'one whose phenotype allows the heritable survival of a given herbicide dose that its respective wild type would find lethal' (Weed Technology 1998). During the 1950's, as chemical weed control grew in prominence, predictions regarding the fate of herbicide use emerged (Harper 1956). The first commercially available herbicide was 2,4-D (as mentioned in section 1.2.2.1). Initial cases of 2,4-D resistance were recorded in 1957, with resistant *Commelina diffusia* (spreading dayflower) being identified in Hawaiian sugar cane fields (Hilton 1957). At the time, cases of resistance such as these were overlooked due to the level of resistance observed (Heap 1997), but the identification of triazine-resistant *Scenecio vulgaris* (common groundsel) in 1968 would gain more recognition (Ryan 1970). Since these first confirmations of resistance, the number of global incidences recorded has risen substantially (Fig. 1.5: Heap, 2015).

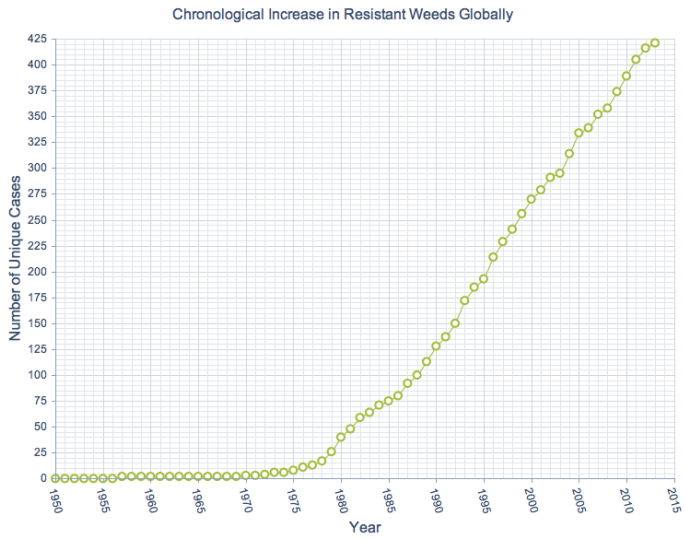
1.4.2 Current status of resistance globally

As of September 2015, there are 246 plant species resistant to one or more of 157 herbicide active ingredients, infesting 86 varieties of crop in 66 countries; of these, 143 are dicotyledonous species and 103 are monocotyledonous (Heap *et al* 2015). There are currently twenty-five herbicide sites of action commercially available; resistance has evolved to twenty-two of these sites of action in one or more weed species. Overall, these figures represent 459 unique cases (the number of species multiplied by the site of action) of herbicide resistant weeds (Figure 1.5a: Heap *et al* 2015).

1.4.3 ALS and ACCase MOA

Herbicides classified within category A of the HRAC classification of biochemical MOA, have a MOA that inhibits the acetyl coenzyme A synthase (ACCase) enzyme. The ACCase molecule is involved in the production of essential long-chain fatty acids; inhibition of the ACCase enzyme causes death resulting from long-chain fatty acid starvation (Délye 2005). The first ACCase herbicide was marketed over 30 years ago, since then three classes of ACCase herbicide; the Aryloxyphenoxypropionates (AOPP's, 'fops'), cyclohexanediones (CHD's, 'dims') and phenlypyrazolines ('dens') have been developed (HGCA 2010). 47 weed species have been identified as exhibiting resistance to ACCase MOA (Figure 1.5b) (Heap *et al* 2015).

(a)



(b)

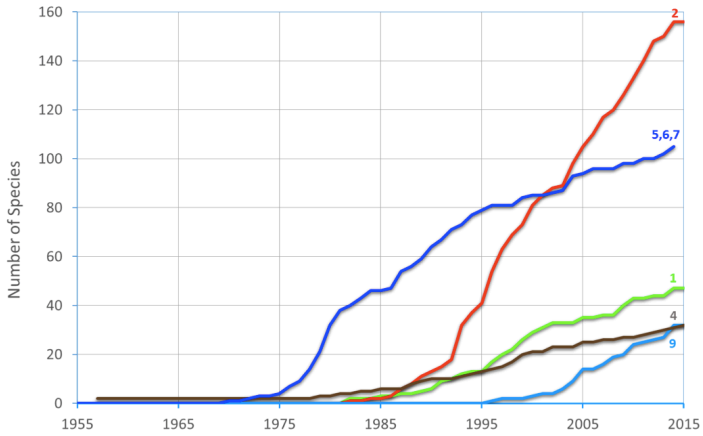


Figure 1.5: (a) A chronology of the number of unique cases of herbicide resistance recorded globally between 1950 and 2014 (b) number of species resistant to ALS (red), ACCase (green) MOA, EPSPS inhibitors (light blue), synthetic auxins (brown) and PSII inhibitors (dark blue) (Source: Heap *et al* 2015).

Herbicides classified within category B of the HRAC classification of biochemical MOA, have a MOA that inhibits the acetolactate synthase (ALS) enzyme (Tranel and Wright 2002). The ALS molecule is an anabolic enzyme that catalyzes a reaction in the initial stages of the biosynthesis of the branch chained amino acids leucine,

isoleucine and valine (Tranel and Wright 2002). The inhibition of the ALS enzyme results in plant mortality as a result of limited availability of leucine, isoleucine and valine, disruption of protein synthesis, and accumulation of 2-ketobutyrate (Yu and Powles 2014).

The first sulfonylurea ALS herbicide (chlorsulfuron) was introduced in 1982. Herbicides with an ALS MOA were revolutionary when first introduced, as they were highly active, requiring only low field application rates (grams/hectare) of active ingredient, reducing overall herbicide inputs (Yu and Powles 2014). Since their first introduction, five classes of ALS herbicide, the imidazolinones, sulfonylureas, sulfonylamino-carbonyl-triazolinones, pyrimidinyl(thio)benzoates, and triazolopyrimidines have been developed (HGCA 2010). 157 weed species have been identified as exhibiting resistance to ALS MOA (Figure 1.5b) (Heap *et al* 2015).

1.4.4 Herbicide resistance evolution

In comparison to classical cultural techniques, herbicides are relatively inexpensive and highly efficacious. Continuous application of a single herbicide MOA in a homogeneous environment (free from cultural management), is an attractive arable paradigm practiced globally. However, this practice promotes the evolution of herbicide resistance.

Weed populations typically contain a high level of genetic variability (Cavan *et al* 2000). There is the potential for herbicide resistance conferring polymorphisms to occur in genes that encode herbicide target enzymes, and/or variation in enzyme

families that have the capacity to reduce the concentration of herbicide active ingredient reaching the SOA. Herbicide application selects for these initially rare mutations that confer herbicide resistance. Sequential herbicide application increases the frequency of mutations to a point where a large proportion of the population is no longer susceptible to the herbicide (Neve 2007). Each mutation confers a particular resistance mechanism or contributes to it; there are a number of potential mechanisms, but all can be broadly be defined as conferring either a target-site resistance (TSR) or non-target-site resistance (NSTR).

1.5 Mechanisms of resistance

1.5.1 Target site resistance

The term 'target-site resistance (TSR) mechanism' encompasses all mechanisms of herbicide resistance that modify the herbicide SOA to prevent effective herbicide binding. Target-site resistance can affect the SOA in one of two ways: (1) via mutations of the target enzyme that prevent normal herbicide-target binding, preventing inhibition by the herbicide; (2) via various mechanisms that result in over-expression of the target enzyme, so that complete inhibition cannot be achieved (Powles and Yu 2010).

The first type of target-site mechanism is mutations that alter the amino acid sequence of the target enzyme result from rare/random *de novo* single-nucleotide polymorphisms (SNPs). These mutations enhance plant survival and fitness following herbicide exposure and are selected by herbicide application.

The second form of target-site resistance, overexpression of the target enzyme, can occur as a result of two mechanisms: up-regulation of the target-site or target-site gene amplification. Up-regulation of the target-site occurs when the genes that control the expression of the target enzyme are mutated to cause the target-site to be over-expressed. Target-site over-expression increases the number of target enzyme molecules produced to a level where the applied herbicide dose is insufficient to lethally inhibit enzyme function (Jasieniuk *et al* 1996).

Target-site gene amplification has the same outcome as up-regulation of the target-site (over production of the target-site enzyme), but the mechanism by which this phenomenon occurs is different. Overexpression of the target enzyme as a result of target-site gene amplification, results from an increase in the number of genes that encode the target-site within the genome. This increase in target-site gene number can occur as a result of retrotransposition, a change in ploidy, or replication slippage, for example. Target-site gene amplification resulting from a transposon or RNA-mediated mechanism was first identified in Glyphosate resistant *Amaranthus palmeri* in the USA (Gaines *et al* 2010).

1.5.2 Non target-site resistance

Non-target-site resistance (NTSR) mechanisms, unlike target-site resistance, do not directly involve modification, over-expression or amplification of the herbicide SOA. Instead, NTSR mechanisms reduce the concentration of herbicide active ingredient reaching the target-site. Non target-site resistance can take three main forms (which may act together to produce the NTSR phenotype), these are: (1) enhanced metabolism (EMR), where the herbicide is detoxified by catabolic

enzymes into non-toxic compounds before it reaches the target-site; (2) sequestration, where the herbicide is sequestered into the vacuole of the plant so it cannot be effective; and (3) impaired absorption/translocation, where the movement of the herbicide through the plant to the target enzyme in question is reduced or prevented (Délye 2012).

Although the precise genetic basis of NTSR mechanisms is usually unknown, resistance endowed via these mechanisms is considered to be quantitatively inherited trait. Theoretically, the evolutionary dynamics of polygenic trait selection differs from selection of monogenic traits, with the former being selected from a populations standing genetic variation, as opposed to *de novo* mutations (section 1.5.1) (although the importance of standing genetic variation vs. *de novo* mutations in the selection of NTSR is still unclear) (Délye *et al* 2013). Standing genetic variation is the natural allelic variation that exists between individuals within a population. Some alleles present within a population implicated in NTSR may provide a low level of resistance that is dose-dependent. Individuals that survive herbicide application as a result of low resistance, dose-dependent alleles reproduce post-selection, recombining to create new gene combinations that increase the dose-dependent expression of the non-target-site mechanism in the next generation (Délye 2012).

Currently, the precise genes involved in enhanced metabolic resistance are unknown. However, recently Cummins *et al* (2013) identified the *AmGSTF1* gene in *Alopecurus myosuroides*. While not involved directly in metabolization, when present, the gene appears to regulate a plants ability to metabolise the PSII inhibiting

herbicides atrazine (chloro-s-triazine) and chlorotoluron (phenylurea). Similarly, Gaines *et al* (2014) found that when experimentally evolved lines of *Lolium rigidum* were selected with diclofop, four genes (two P450 Cytochrome mono-oxygenases, one nitrogen mono-oxygenase, and a Glutathione sythanse) exhibited consistent expression, aiding in the expression of a metabolically derived diclofop resistant phenotype.

1.6 Herbicide resistant *A. myosuroides* in the UK

1.6.1 Current frequency of resistance

The first case of *A. myosuroides* herbicide resistance (to the photosystem II inhibiting herbicide chlorotoluron) was recorded in the UK at a farm in Faringdon, Oxfordshire (England) in 1982 (Moss and Cussans 1985). Since then, *A. myosuroides* populations resistant to ACCase, ALS, and microtubule inhibiting MOA have been identified throughout Europe: particularly in *A. myosuroides'* main distribution of the UK, France, Belgium, Netherlands, and Germany (Table 1.3) (Moss *et al* 2007; Heap 2015). In the UK alone, it is estimated that resistance to one or more of these MOA is present on more than 16,000 farms across 34 counties (Figure 1.6) (Hull *et al* 2014), although estimates of the area infested with *Alopecurus myosuroides* have not been published. Included in this are cases of cross-resistance (resistance to several different chemical classes represented by one SOA/biochemical MOA (Hall *et al* 1994)) and multiple-resistance (resistance to several chemical classes represented by several SOA/biochemical MOA. (Cocker *et al* 1999; Powles *et al* 2010; Heap 2015) to the two most important post-emergence herbicides for managing *A. myosuroides* in UK winter cereals: ACCase and ALS inhibitors.

Table 1.3: Cases of resistant recorded across *A. myosuroides'* main European distribution. These countries are highlighted in Figure 1.3 (Source: Heap *et al* 2015).

Country	Year	Active Ingredient	Site of Action
Belgium	1996	clodinafop-propargyl, fenoxaprop-P-ethyl	ACCase inhibitors
		isoproturon	PSII inhibitor (Ureas and amides)
		atrazine, chlorotoluron, clodinafop-propargyl, fenoxaprop-P-ethyl, flupyrsulfuron-methyl-sodium, pendimethalin, propaquizafop	**Multiple Resistance** ACCase inhibitors ALS inhibitors Photosystem II inhibitors PSII inhibitor (Ureas and amides) Microtubule inhibitors
France	1983	clodinafop-propargyl, cycloxydim, diclofop-methyl, fenoxaprop-P-ethyl, haloxyfop-P-methyl, sethoxydim	ACCase inhibitors
	2006	imazamethabenz-methyl, iodosulfuron-methyl-sodium, mesosulfuron-methyl	ALS inhibitors
Germany	1983	chlorotoluron, fenoxaprop-P-ethyl, isoproturon	**Multiple Resistance:** ACCase inhibitors PSII inhibitor (Ureas and amides)
	2001	flupyrsulfuron-methyl-sodium	ALS inhibitors
	2003	clethodim, cycloxydim, fenoxaprop-P-ethyl, fluazifop-P-butyl	ACCase inhibitors
	2007	chlorotoluron, fenoxaprop-P-ethyl, lufenacet, isoproturon, mesosulfuron-methyl, pinoxaden	**Multiple Resistance:** ACCase inhibitors ALS inhibitors PSII inhibitor (Ureas and amides) Long chain fatty acid inhibitors
	2009	cycloxydim, fenoxaprop-P-ethyl, flupyrsulfuron-methyl-sodium, mesosulfuron-methyl, pinoxaden	**Multiple Resistance:** ACCase inhibitors ALS inhibitors
Netherlands	1989	chlorotoluron	PSII inhibitor (Ureas and amides)
	1996	chlorotoluron, clodinafop-propargyl, fenoxaprop-P-ethyl, isoproturon	**Multiple Resistance:** ACCase inhibitors PSII inhibitor (Ureas and amides)
	1999	cycloxydim	ACCase inhibitors
United Kingdom	1982	clodinafop-propargyl, cycloxydim, diclofop-methyl, fenoxaprop-P-ethyl, fluazifop-P-butyl	ACCase inhibitors
		chlorotoluron	PSII inhibitor (Ureas and amides)
	1984	chlorsulfuron, imazamethabenz-methyl, iodosulfuron-methyl-sodium, mesosulfuron-methyl, propoxycarbazone-sodium, pyroxsulam	ALS inhibitors
	1987	pendimethalin	Microtubule inhibitors

Figure 1.6: Cases of resistant *A. myosuroides* recorded across the UK. Counties highlighted in orange have confirmed cases of *A. myosuroides* resistance to at least one MOA: PSII, ACCase, ALS, or microtubule inhibitors. In each county not all fields – sometimes the number may be few - showed resistance. (Source: Hull *et al* 2014).

1.6.2 ACCase herbicides and ACCase resistance in A. myosuroides

The first case of *A. myosuroides* resistance to ACCase MOA was recorded in 1982 in the UK (Heap 2015). *A. myosuroides* populations that exhibit phenotypic resistance to ACCase MOA can be endowed by single nucleotide polymorphisms (SNP) in the ACCase gene. SNP's occurring at one of five nucleotide positions have been shown to give rise to amino acid substitutions at positions Ile-2027, Ile-1781, Trp-2041, Asp-2078 & Gly-2096 of the ACCase gene (Table 1.4) (Délye 2005). As discussed above (section 1.5.2), the genetic causes of ACCase enhanced metabolism are still largely unknown. With ACCase target-site mutations being a relatively small

fraction of the total genes involved in ACCase resistance (Délye *et al* 2010), enhanced metabolism is regarded as a far more important mechanism of ACCase resistance, being estimated to be present in >75% of plants analysed from 243 French *A. myosuroides* populations (Délye *et al* 2007). However, the frequency of enhanced metabolism within a population will depend on the location and field management history.

Table 1.4: ACCase target-site mutations. Nucleotide changes (column 2 for each mutation) that cause an amino acid substitution are underlined, with their corresponding amino acid change shown in column. Wildtype amino acids and their corresponding codon are highlighted in red.

1781		2027		2041		2078		2096	
1	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
Ile	ATA	Trp	TGG	Ile	ATT	Asp	GAT	Gly	GGT
Leu	CTA	Cys	TGC	Val	GTT	Gly	GGT	Ala	GCT
Leu	TTA	Cys	TGT	Asn	AAT				
Val	GTA								
Thr	ACA								

1.6.3 ALS herbicides and A. myosuroides ALS resistance

The first case of *A. myosuroides* resistance to ALS herbicides was recorded in 1984 in the UK (Heap *et al* 2015). To date, there have been two amino acid positions identified in the ALS gene of *A. myosuroides* where a mutation endows target-site resistance to ALS inhibiting herbicides (Table 1.5) (Tranel and Wright 2002; Heap 2014a). As discussed above (section 1.5.2), the genetic causes of ALS enhanced metabolism are still largely unknown.

Table 1.5: ALS target-site mutations. Nucleotide changes (column 2 for each mutation) that cause an amino acid substitution are underlined, with their corresponding amino acid change shown in column. Wildtype amino acids and their corresponding codon are highlighted in red.

197		574	
1	**2**	**1**	**2**
Pro	CCC	Trp	TGG
Ala	GCC	Leu	TTG
Thr	ACC		
Ser	TCC		
His	CAC		
Leu	CTC		
Arg	CGC		

1.7 Integrated weed management (IWM) practices to control resistance

In light *A. myosuroides* resistance to essential ALS and ACCase herbicide MOA recorded in the UK and northern Europe, it is important that *A. myosuroides* management no longer relies solely upon chemical control measures. This is compounded by the fact that fewer herbicides are available due to an increase in EU legislation, such as directives EC/1107/2009 (which replaced 91/414/EEC: concerning the placing of plant production products on the market) and the Water Framework Directive (2000/60/EC) (Stark 2011).

A lack of new herbicide MOA being brought to the commercial market (Lutman *et al* 2013) puts greater emphasis on integrated weed management (IWM). IWM combines cultural and rotational practices, an effective chemical control programme, and an effective resistance management plan to control the size of *A. myosuroides* populations (HGCA 2010: Bayer Crop Science 2012). In line with this, the Sustainable Use Directive (2009/128/EC) states that from 2014, farmers will have to place greater emphasis on non-chemical control measures to reduce pesticide inputs (Stark 2011).

IWM incorporates the use of pre-emergent herbicides, ploughing, autumn-sown break crops (e.g. oilseed rape (*Brassica napus*)), spring cropping, and delayed drilling to reduce weed populations (Lutman et al 2013). Many studies have been conducted to show the effectiveness of each of these management strategies, with spring cropping estimated to reduce *A. myosuroides* infestations up to 80%, ploughing by 67%, and delayed drilling by 37% (Lutman *et al* 2013). Including effective chemical control strategies into IWM relies on understanding how resistance and resistance mechanisms evolve under different chemical management practices.

1.8 Objectives

This thesis aims to establish the frequency and distribution of herbicide resistance in a collection of UK *Alopecurus myosuroides* populations, as well as the factors that drive the evolution of herbicide resistance in *A. myosuroides*. This research will centre upon two post-emergent modes of action (MOA) that are imperative to *A. myosuroides* control in the UK at present: acetolactate synthase (ALS) and acetyl co-enzyme A carboxylase (ACCase). An initial survey of *A. myosuroides* populations from a core arable region of the UK will be conducted. From this survey, the extent of ALS and ACCase resistance will be evaluated and the mechanism(s) endowing resistance will be determined. Based on this analysis, several populations with contrasting phenotypic and mechanistic frequencies of ALS and ACCase resistance will be identified for annual resampling (2012 - 2014). Resampled populations will be examined to establish inter-annual changes in the frequency of ALS and ACCase resistance phenotypes and mechanisms. These changes will be related to documented weed management so that relationships between resistance frequency, mechanism

frequency, and weed management can be established. This will further our understanding of how weed management effects the selection and dynamics of specific ALS and ACCase resistance mechanisms. Additional insight into these dynamics will be gained through the development, application and validation (using data generated in this study) of a model of herbicide resistance evolution.

A number of experiments to analyse the fitness and selection dynamics of certain ALS resistance mechanisms will be conducted, investigating how these attributes have the potential to effect change in the frequency of the resistance mechanism in question. These areas of research will include; determining the segregation of TSR mutations that occur at position the Pro-197 within the acetolactate synthase (ALS) gene, and establishing how the presence of existing ACCase enhanced metabolism effects the subsequent rate of ALS enhanced metabolism selection within populations of *A. myosuroides*.

2.0 Establishing the extent of ACCase and ALS herbicide resistance and herbicide resistance mechanisms in UK populations of *Alopecurus myosuroides.*

2.1 Introduction

2.1.1 Herbicide Resistant Alopecurus myosuroides

In France, Germany, Belgium, the Netherlands, and the UK, sustainably maintaining the yield of winter cereals is under threat from the grass weed *Alopecurus myosuroides*. Herbicides predominate *A. myosuroides* management; of particular importance are those with post-emergent acetolactate synthase (ALS) and acetyl co-enzyme A carboxylase (ACCase) modes of action (MOA) (Moss 2013). The repeated annual application of these modes of action and lack of diversity in the integrated weed management (IWM) - i.e. simplification of rotation systems and the introduction of low or no tillage (Lutman *et al* 2013) - has resulted in widespread evolution of resistance in *A. myosuroides* (Délye *et al* 2010). Currently, *A. myosuroides* resistant to at least one MOA has been identified in fourteen European countries, with ACCase resistance (11 countries) and ALS resistance (9) being most common (Heap 2015). Endowed by either or both mechanisms of target-site resistance (TSR) or enhanced metabolism (EMR), resistance reduces the capability of ACCase and ALS herbicides to control *A. myosuroides*, exacerbating population sizes, culminating in detrimental effects on crop yield (HGCA 2010).

2.1.2 Occurrence of ALS and ACCase A. myosuroides resistance

Surveys conducted within *A. myosuroides'* key distribution consistently establish the

presence of ACCase resistance within 80–100% of sampled populations (Moss and Perryman 2007; Délye *et al* 2007; Délye *et al* 2010; Hess *et al* 2012; Hull *et al* 2014). Every population tested from three regions of Germany exhibited resistance to fenoxaprop-p-ethyl (Hess *et al* 2012); of sixty-six populations from France, 78.7% and 54.6% contained >50% of individuals resistant to fenoxaprop and clodinafop respectively (Délye *et al* 2010); in the UK eighty-percent of 25 randomly surveyed farms exhibited resistance to fenoxaprop-p-ethyl (Moss *et al* 2007), while eighty-four percent of 122 non-randomly sampled populations exhibited resistance to cycloxydim (Hull *et al* 2014).

ALS resistance exhibits greater regional variation than ACCase resistance. In the Côte d'or region of France, 121/124 (98%) *A. myosuroides* populations sampled exhibited resistance to flupyrsulfuron, with 50% of all plants tested being resistant (Chauvel *et al* 2006). In Germany, Petersen (2011) identified resistance to the ALS herbicides in approximately 6% of 236 populations tested, while Hess *et al* (2012) recorded mesosulfuron-methyl + iodosulfuron-methyl-sodium resistance in 4%, 26% and 60% of fields tested respectively from three regions studied. In the UK, resistance to mesosulfuron-methyl + iodosulfuron-methyl-sodium has been estimated to be present on over 700 farms across 27 counties (Hull *et al* 2014). A region's herbicide selection history, the number of populations studied, and the way in which the populations are sampled (e.g. random Vs. non-random) will all be contributing factors in the levels of ACCase and ALS herbicide resistance identified.

2.1.3 Distribution of ALS and ACCase resistance mechanisms

Consistently, target-site mutations that endow resistance to ACCase MOA at position

Ile-1781 of the ACCase gene are found to occur most frequently in comparison to the four other ACCase target-site mutations (Table 2.1).

Table 2.1: Percentage of confirmed resistant populations that have at least one individual with an ACCase target-site mutations from a number of publications. The reported percentage of populations containing an Ile-1781, Trp-2027, Ile-2041, Asp-2078 and Gly-2096 ACCase target-site mutation from a number of publications for France, Germany and the UK.

ACCase Mutation	Country	Percent (%) of confirmed resistant populations that have at least one individual with this mutation	Number Populations Sampled	Author
Ile-1781	France	34.5	116	Menchari *et al* 2006
	France	41.4	116	Délye *et al* 2007
	France UK Germany	40.9 9.4 81.4	66 75 86	Délye *et al* 2011
	Germany	55 70 66	28 42 21	Hess *et al* 2012
Trp-2027	France	33.6	116	Menchari *et al* 2006
	France	17.2	116	Délye *et al* 2007
	France UK Germany	28.8 25.5 8.0	66 75 86	Délye *et al* 2011
	Germany	25 75 79	28 42 21	Hess *et al* 2012
Ile-2041	France	12.9	116	Menchari *et al* 2006
	France	15.5	116	Délye *et al* 2007
	France UK Germany	28.8 29.1 12.0	66 75 86	Délye *et al* 2011
	Germany	65 40 28	28 42 21	Hess *et al* 2012
Asp-2078	France	14.7	116	Menchari *et al* 2006
	France	14.7	116	Délye *et al* 2007
	France UK Germany	16.7 16.3 5.3	66 75 86	Délye *et al* 2011
	Germany	55 15 83	28 42 21	Hess *et al* 2012
Gly-2096	France	13.8	116	Menchari *et al* 2006
	France	14.7	116	Délye *et al* 2007
	France UK Germany	31.8 23.8 18.7	66 75 86	Délye *et al* 2011
	Germany	10 55 10	28 42 21	Hess *et al* 2012

In contrast, the prevalence and extent of ALS target-site mutations is less well established. Hess *et al* (2012) found that of 32, 51 and 21 populations sampled from

three regions of Germany, Pro-197 TSR mutations were identified in 0%, 31% and 20%, and Trp-574 mutations were identified in 0%, 0% and 55% of populations, respectively. In the UK, of 570 plants from 19 random populations sampled between 2009 and 2011, 7% possessed Pro-197-Thr target-site mutations, and 7% possessed Trp-574-Leu target-site mutations (Moss *et al* 2014).

The level of ACCase and ALS enhanced metabolism is largely unknown. By, comparing ACCase sensitivity assays (24,300 seedlings) to ACCase TSR genotyping data (13,188 seedlings) from 243 populations, it was estimated that 75% of plants tested were resistant via ACCase enhanced metabolism (Délye *et al* 2007). 80% of 53 Danish *A. myosuroides* populations studied exhibited some degree of fenoxaprop-p-ethyl metabolism (Keshtkar *et al* 2015). In the UK, 20% of 570 plants from 19 random populations sampled between 2009 and 2011 survived treatment with an ALS herbicide through a mechanism of non target-site resistance (Moss *et al* 2014).

2.2 Objectives

Work reported in this chapter aims to determine the presence and extent of post-emergent ALS and ACCase herbicide resistance in populations of *A. myosuroides* from an important arable region of the UK. Following field collections of 92 populations in 2011, glasshouse dose response assays were performed to establish the proportion of each population that exhibited phenotypic resistance to the ALS herbicide mesosulfuron-methyl + iodosulfuron-methyl-sodium and the ACCase herbicide fenoxaprop-p-ethyl. Subsequently, a series of molecular genetic and biochemical assays were performed to determine the target-site and enhanced metabolism mechanisms that endow resistance in each of the ninety-two populations.

Seed was collected from 46-paired populations, each from a different farm, one from a field treated with the ALS herbicide mesosulfuron-methyl + iodosulfuron-methyl-sodium and one from a field not treated with mesosulfuron-methyl + iodosulfuron-methyl-sodium in the year of sampling, to determine the effect that herbicide treatment in the year of sampling on the estimation of resistance frequency.

2.3 Materials and Methods

2.3.1 Sample Collection

In 2011, forty-six UK farms were selected for sampling by Bayer CropScience (UK). These farms were chosen from counties in which resistance to ACCase and ALS modes of action (MOA) had already been confirmed, and from farmers who had expressed that herbicidal control of *A. myosuroides* could be problematic. This non-random sampling approach was adopted, as it was the prevalence of ALS and ACCase resistance and resistance mechanisms in areas in which resistant *A. myosuroides* is frequent that was of interest. A random sampling of UK *A. myosuroides* could have been employed, including populations from Scotland for example, but this may have led to an underestimate of resistance in key areas. The presence of ACCase and ALS resistance on each of the sampled farms was previously unknown. From each of the forty-six farms, two fields had a sample of *A. myosuroides* seed collected (a total of ninety-two fields) (Figure 2.1). Both fields sampled from each farm have a long history of herbicide use, however in the year of sampling, one field had been treated with the ALS herbicide mesosulfuron-methyl + iodosulfuron-methyl-sodium (including the safener mefenpyr di-ethyl) (commercial name Atlantis WG (Bayer CropScience)) and one field that had not been treated with mesosulfuron-methyl + iodosulfuron-methyl-sodium during the sampling year.

Figure 2.1: The location of UK farms from which *A. myosuroides* populations were sampled in 2011. Each point represents one farm. Points are marked as near to the GPS location of the farm as possible, without obstructing other points.

This sampling strategy was employed to evaluate the effect that mesosulfuron-methyl + iodosulfuron-methyl-sodium application in the year of sampling has on the frequency of mesosulfuron-methyl + iodosulfuron-methyl-sodium resistance. (The two fields from each farm were not analysed as pairwise observations. Although they came from the same farm and could have been managed similarly by the farmer, initial analysis revealed that there was no relationship between fields sampled from the same farm). Herbicide treatment in the year of seed collection removes the majority of emerged susceptible individuals from the population, leading to an overestimate of resistance frequency at the population level as estimates of resistance frequency are based on seeds produced by surviving (and therefore predominantly resistant) individuals and do not take account of the frequency of susceptible individuals within the seed bank. Sampling populations that are not treated with ALS herbicides in the year of seed collection is able to give a more accurate prediction of the frequency of ALS resistance within the entire population.

To collect a sample of *A. myosuroides* seed from all forty-six pairs of fields, a Bayer CropScience (UK) representative visited each farm during July 2011 when *A. myosuroides* seed was mature. The tramlines in each field were walked for the entire field. When individual plants or patches of *A. myosuroides* were encountered, seed of one or two seed-heads were collected, to ensure that the sample was random and representative of the *A. myosuroides* population present. Seeds were sampled from a variety of crops: winter wheat (*Triticum aestivum* (55 fields)), winter beans (*Vicia faba* (18)), sugar beet (*Beta vulgaris* (6)), summer beans (*V. faba* (3)), spring barley (*Hordeum vulgare* (3)), winter oilseed rape (*Brassica napus* (1)), winter oats (*Avena sativa* (1)), maize (*Zea mays* (1)), crop unrecorded (5). Cultural weed management techniques, pre-emergent and post-emergent herbicide treatments (other than treatment with mesosulfuron-methyl + iodosulfuron-methyl-sodium) were not recorded at the time of collection.

2.3.2 Determining resistance phenotype

All ninety-two populations (as well as one susceptible (R35) and one resistant (R36) standard *A. myosuroides* population (provided by Bayer CropScience) to test the efficacy of herbicide application as a negative and positive control respectively) were phenotyped to identify the presence of resistance to one ALS- and one ACCase-inhibitor product. Into each of six 8x8cm jiffy pots containing a soil mixture of loamy silt soil (LSI: 19% sand, 60% silt, 22% clay, 2.2% organic matter: pH 7.4), approximately 25 seeds of one population were sown. Once the seeds were sown, they were covered with approximately 0.5mm of Quartz sand RQ 16 (0.9-2.0 mm). Each population's jiffy pots were divided into pairs, and each pair placed into separate polypropylene tray (L x W x H = 50x25x5cm). One pair of pots was to be

used to identify resistance to the ALS herbicide mesosulfuron-methyl + iodosulfuron-methyl-sodium, one pair of pots was to be used to identify resistance to the ACCase herbicide clodinofop-propargyl, and one pair of pots was to be used as an untreated control as comparison. With each polypropylene tray containing the paired pots of five populations, a total of nineteen trays for each treatment (ALS-, ACCase-inhibitor and control) were required. All population treatments were placed in collection number order (1 to 92) within a climate controlled glasshouse compartment. The glasshouse (temperature: 22.0°C/16°C; photoperiod: 14 hours; humidity: 50%) was equipped with supplementary lighting (turned on when natural light intensity < 15 kilolux (bulbs: Philips Son-T Agro (400 W)) and an automatic shading system (activated when the light intensity exceeds 55 kilolux) was used. Trays were watered after sowing and at regular intervals until herbicide application.

At the two-leaf stage, plants were phenotyped for ALS resistance using the UK recommended field dose of mesosulfuron-methyl (14g a.i ha^{-1}) + iodosulfuron-methyl-sodium (2.4g a.i ha^{-1}) with 280g a.i. ha^{-1} of genapol LRO fluid adjuvant, or the UK recommended field dose of ACCase herbicide using 60g a.i ha^{-1} clodinofop-propargyl with 842g a.i ha^{-1} actirob b adjuvant. Herbicide was applied at a volume of 300 l ha^{-1} and a pressure of 200 kPa using a track sprayer fitted with a Teejet XR8002 nozzle. The nozzle was positioned approximately 35 – 40 cm from the median plant height. After herbicide application, 5-6 ml (equivalent to approximately 100 kg N ha^{-1}) of the liquid fertilizer (Wuxal Super: N8 – P8 – K6, citrus fertilizer) was applied. Twenty-one days after herbicide application, the resistance status of each pot was evaluated using a visual score, based on a scale from 0% (no visible damage or growth reduction of any plants) and 100% (complete mortality of all

plants) (Table 2.2). A mean visual injury rating score was derived from the two pots tested for each population to determine the level of phenotypic resistance in comparison to a predetermined scale: 0 – 49% resistant: 50 – 79% intermediate resistance: 80 – 100% susceptible. These three levels of phenotypic resistance were used as they have been found by Bayer CropScience to correlate with agronomic problems within the field. Susceptible populations will pose no agronomic problem, intermediate populations may pose a problem to the farmer, and resistant populations definitely will pose an agronomic problem in the field if population densities are great enough.

Table 2.2: Details of the visual damage score used to assess resistance (Source: Bayer CropScience, personal communication)

Visual score	Definition of Score
0	No damage, same as untreated control
10	No bleaching and/or inhibition of plant growth from 10 - 20%
20	Light bleaching in leaf apices and/or inhibition of plant growth from 20 – 30%
30	Moderate bleaching and/or inhibition of plant growth from 30 – 40%
40	Foliar necrosis of 20% and/or inhibition of plant growth from 40 – 50%
50	Foliar necrosis of 30% and/or inhibition of plant growth from 50 – 60%
60	Foliar necrosis of 50% and/or inhibition of plant growth from 60 – 70%
70	Foliar necrosis of 60% and/or inhibition of plant growth from 70 – 80%
80	Foliar necrosis of 70% and/or inhibition of plant growth from 80 – 90%
90	Foliar necrosis higher than 80% and/or inhibition of plant growth higher >90%
100	Plant death (100% necrosis)

2.3.3 Target-site analysis

2.3.3.1 DNA Extraction

To identify whether any of the sampled populations were resistant to ACCase and ALS MOA as a result of target-site resistance, polymerase chain reaction (PCR) and Pyrosequencing were used to identify target-site mutations. Mutations at seven nucleotide positions, two ALS and five ACCase (as described in section 1.6.2 and 1.6.3) were studied. Before herbicide application (section 2.3.2), a 2cm section of leaf tissue was excised from eight plants per population. Each piece of leaf tissue was placed into a separate well of a 96-deep-well plate. To each well a stainless steel bead and 400µL of extraction buffer (100mM Tris(HCl) and 1 M KCl, pH 9.5) were added. Plates were shaken (30rpm for 10 minutes) using a Qiagen TissueLyser II, and centrifuged (4000rpm for 10 minutes) using a Sigma laboratory 4K15C centrifuge to extract the DNA. 100µl of supernatant was transferred into a new 96-well plate, from which a second plate containing 5µl supernatant and 250µl of Sigma-Aldrich W3500 sterile filtered tissue culture water was produced. The 100µl plate was stored at -80°C, while the 5µl plate was used as the PCR DNA template.

2.3.3.2 PCR Conditions for identifying ALS TSR mutations

To identify single nucleotide polymorphisms (SNP) at position Pro-197 of the ALS gene, a 140bp biotinylated fragment was amplified using forward and reverse primers (forward: 5'-GTGCTACCAACCTCGTCTC-3'; reverse: 5'-GGAGCGGGTGACCTCTACAAT-3'). A Master mix consisting of 10µl forward primer, 10µl reverse primer, 1030µl Sigma-Aldrich W3500 sterile filtered tissue culture water and 2500µl of Taq polymerase was produced. 5µl of DNA template and 20µl master mix was combined in a PCR plate (total volume = 25µl) using a

Beckman Coulter Biomek 3000 workstation. The plate was sealed and centrifuged using an Eppendorf 5810R centrifuge at 4000rpm for 1 minute. Amplification of the target region was carried out using an Eppendorf Mastercycler Gradient PCR machine set to denature at 94°C for 30s for 45 cycles, anneal at 60°C for 30s for 45 cycles, and elongate at 72°C for 40s for 45 cycles. PCR products were stored at 4°C until use.

To identify single nucleotide polymorphisms (SNP) at position Pro-574 of the ALS gene, a 401bp biotinylated fragment was amplified using forward and reverse primers (forward: 5'-ATTCAGGAGTTGGCACTGATT-3'; reverse: 5'-AGCTCTTGCCGAAGTTCTGAT-3'). A Master mix consisting of 10µl forward primer, 10µl reverse primer, 1030µl Sigma-Aldrich W3500 sterile filtered tissue culture water and 2500µl of Taq polymerase was produced. 5µl of DNA template and 20µl master mix was combined in a PCR plate (total volume = 25µl) using a Beckman Coulter Biomek 3000 workstation. The plate was sealed and centrifuged using an Eppendorf 5810R centrifuge at 4000rpm for 1 minute. Amplification of the target region was carried out using an Eppendorf Mastercycler Gradient PCR machine set to denature at 94°C for 30s for 45 cycles, anneal at 54.2°C for 30s for 45 cycles, and elongate at 72°C for 40s for 45 cycles. PCR products were stored at 4°C until use.

2.3.3.3 PCR Conditions for identifying ACCase TSR mutations

To identify single nucleotide polymorphisms (SNP) at position Ile-1781 of the ACCase gene, a 182bp biotinylated fragment was amplified using forward and reverse primers (forward: 5'-GCACACAAGATGCAGCTAGATAGT-3'; reverse:

5’- TCCGATTCCAACAGTTCGT-3’). A Master mix consisting of 10µl forward primer, 10µl reverse primer, 1030µl Sigma-Aldrich W3500 sterile filtered tissue culture water and 2500µl of Taq polymerase was produced. 5µl of DNA template and 20µl master mix was combined in a PCR plate (total volume = 25µl) using a Beckman Coulter Biomek 3000 workstation. The plate was sealed and centrifuged using an Eppendorf 5810R centrifuge at 4000rpm for 1 minute. Amplification of the target region was carried out using an Eppendorf Mastercycler Gradient PCR machine set to denature at 94°C for 30s for 45 cycles, anneal at 54.0°C for 30s for 45 cycles, and elongate at 72°C for 40s for 45 cycles. PCR products were stored at 4°C until use.

To identify single nucleotide polymorphisms (SNP) at position identify Trp-2027, Ile-2041, Asp-2078, Gly-2096 of the ACCase gene, a 481bp biotinylated fragment was amplified using forward and reverse primers (forward: 5’-TCCTGTTGGTGTTATAGCTG-3’; reverse: 5’-GGATCAAGCCTACCCATGCA-3’). A Master mix consisting of 10µl forward primer, 10µl reverse primer, 1030µl Sigma-Aldrich W3500 sterile filtered tissue culture water and 2500µl of Taq polymerase was produced. 5µl of DNA template and 20µl master mix was combined in a PCR plate (total volume = 25µl) using a Beckman Coulter Biomek 3000 workstation. The plate was sealed and centrifuged using an Eppendorf 5810R centrifuge at 4000rpm for 1 minute. Amplification of the target region was carried out using an Eppendorf Mastercycler Gradient PCR machine set to denature at 94°C for 30s for 45 cycles, anneal at 54.1°C for 30s for 45 cycles, and elongate at 72°C for 40s for 45 cycles. PCR products were stored at 4°C until use.

2.3.3.4 Pyrosequencing

The PCR product was split into two 12µl aliquots for cleaning and sequencing. 70µl of binding buffer and beads was added to each aliquot and the mixture was shaken for five minutes. 12µl of annealing buffer containing the relevant sequence primer (Table 2.3) were added to each well of a Pyrosequencing plate. The bead bound DNA was washed using a Qiagen Pyromark Q96 workstation in 70% ethanol, sodium hydroxide (NaOH), and wash buffer before being transferred to the sequencing plate and dried at 80°C for two minutes. The Qiagen Pyromark Q96 MD pyrosequencer was set up so that the 10 nucleotides surrounding the single nucleotide polymorphism (SNP) mutation were sequenced. Peaks on the resulting pyrograms indicated the frequency of each nucleotide present.

Table 2.3: Primers used for pyrosequencing of ALS and ACCase target-site mutations.

Site of Action	TSR mutation	Sequencing Primer
ALS	Pro-197	5'-ATGGTCGCTATCACGGGACAGGTT-3'
	Trp-574	5'-CAACATCTGGGAATGGTGGTGCAG-3'
ACCase	Ile-1781	5'-ATGGACTAGGTGTGGAGAAC-3'
	Trp-2027	5'-CCTCTGTTCATACTTGCTAAC-3'
	Ile-2041	5'-GCAAAGAGATCTTTTTGAAGGA-3'
	Asp-2078	5'-GTGGAGGAGCCTGGGTCGTGATT-3'
	Gly-2096	5'-GCTATGCTGAGAGGACTGCAAAG-3'

2.3.4 Analysis of herbicide metabolites

In conjunction with analysing the target-site resistance mechanisms within the sampled populations, high performance liquid chromatography (HPLC) was used to identify herbicide metabolites produced as a result of an enhanced metabolism-based mechanism of resistance. Plants were grown in the glasshouse under the same conditions used for phenotyping (section 2.3.2). Four weeks after sowing, leaf

samples were taken from sixteen plants from each population, eight to test for metabolic resistance to mesosulfuron (ALS) and eight for fenoxaprop (ACCase). ACCase enhanced metabolism was measured using fenoxaprop while ACCase phenotyping was conducted using clodinofop (section 2.3.2). Fenoxaprop was used to identify enhanced metabolism and clodinofop was used to identify phenotype as it is assumed that there is a degree of metabolic cross-resistance between the two herbicides (Beffa, personal communication).

The samples were cut at the join between the first and second leaves and then placed in a 96 deep-well plate with 600µl of water with 400,000 dpm C_{14} labelled mesosulfuron (ALS) or fenoxaprop (ACCase) (activity 4.02 MBq mg−1). The plates were then incubated for 16 hours in an incubator at 28°C so that the herbicide could be absorbed and metabolised by the plant. Leaf samples were then rinsed with water (mesosulfuron) or 80% acetone (fenoxaprop) to remove excess herbicide before being placed into a fresh 96 deep-well plate. To extract and clean the metabolites, 600µl of methanol was added and plates were sealed using a cap-mat, shaken (30rpm for 10 minutes) using a Qiagen TissueLyser II and centrifuged (6000rpm for 10 minutes) using a Sigma laboratory 4K15C centrifuge. The supernatant was then transferred to a 96-square-well plate and dried using a Turbovap® 96 before the extraction process was repeated. The final cleaning stage involved adding 600µl of 80% acetone and 90% acetonitrile for mesosulfuron and fenoxprop respectively, shaking, centrifuging and transferring the supernatant to the 96-square-well plate before drying. The contents of the 96-square-well plate were then re-suspended with 200µl of either 80% acetone (mesosulfuron) or 90% acetonitrile (fenoxaprop) before heating in a Bandelin Sonorex Super RK 156H ultrasonic bath for 5 minutes. The

contents of the 96-well square plate was then transferred to a multi-screen solvent filter plate on a round-bottomed 96-well PP-micro plate, and filtered via centrifugation (2200rpm for 10 minutes).

The herbicide metabolites present within each well of the plates were then measured by HPLC using was a JASCO XL-C 3159 AS with a Phenomenex Kinetex 2.6 μm C18 100A column. 80μl of sample was injected for analysis: the separation gradient consisted of 0.05% H_3PO_4 and 0.05% acetonitrile. The gradient consisted of 1 min at 80% of H_3PO_4, followed by 1.5min of H_3PO_4 decreasing to 25%, and then 8min decreasing to 0%. The radioactivity of the sample was measured using Raytest Miranda detector, and the percentage of parent material metabolised and parent material remaining was calculated for each plant.

2.3.5 Statistical analysis of results

2.3.5.1 Comparing ALS and ACCase genotype to Hardy-Weinberg equilibrium

Due to the small number of individuals sampled in each population, determining Hardy-Weinberg equilibrium within each of the populations ALS or ACCase target-site mutations was not possible. However, analysis across populations containing ALS and ACCase target-site mutations was possible. For each ALS (Pro-197 and Trp-574) and ACCase (Ile-1781, Trp-2027, Ile-2041 Asp-2078 and Gly-2096) target-site mutation, the number of populations containing at least one mutation was identified. Within these mutation containing populations, the number of individuals with wildtype, heterozygous and homozygous alleles was statistically compared using a Fisher's exact test in R (R Development Core Team 2012) to the 1:2:1

wildtype, heterozygous and homozygous ratio expected if the populations are in Hardy-Weinberg equilibrium (1:2:1).

2.3.5.2 Comparing ALS and ACCase phenotypic resistance

The correlation between the occurrence of ALS and ACCase susceptible, intermediate and resistant phenotypes within populations was assessed. For this, the ninety-two *A. myosuroides* samples were divided into those treated with mesosulfuron-methyl + iodosulfuron-methyl-sodium in the year of sampling and those untreated with mesosulfuron-methyl + iodosulfuron-methyl-sodium in the year of sampling. The number of mesosulfuron-methyl + iodosulfuron-methyl-sodium treated populations phenotypically susceptible, intermediate and resistant to ALS MOA were compared to the number of mesosulfuron-methyl + iodosulfuron-methyl-sodium treated populations phenotypically susceptible, intermediate and resistant to ACCase MOA using a Fisher's exact test in R (R Development Core Team 2012). The same analysis was conducted for mesosulfuron-methyl + iodosulfuron-methyl-sodium untreated samples. If a non-significant result were observed, this would be an indication that phenotypic resistance to ALS and ACCase inhibitors is correlated with one another within a population.

2.3.5.3 Comparing ALS and ACCase target-site resistance

The correlation in the proportion of ALS target-site resistance and ACCase target-site resistance within populations was assessed. For this, the ninety-two *A. myosuroides* samples were divided into mesosulfuron-methyl + iodosulfuron-methyl-sodium treated and mesosulfuron-methyl + iodosulfuron-methyl-sodium untreated populations. In each population, the proportion of the eight plants tested possessing

an ALS target-site mutation (either Pro-197 and Trp-574) and the proportion of the eight plants tested possessing an ACCase target-site mutation (either Ile-1781, Trp-2027, Ile-2041 Asp-2078 and Gly-2096) was calculated. The correlation between the proportions of ALS and ACCase target-site mutations was evaluated using a generalized linear model in R (R Development Core Team 2012), incorporating mesosulfuron-methyl + iodosulfuron-methyl-sodium treated and mesosulfuron-methyl + iodosulfuron-methyl-sodium untreated populations as a factor.

2.3.5.4 Comparing ALS and ACCase enhanced metabolism

Using HPLC analysis, the amount of radiolabelled herbicide that remained (%_remaining) un-metabolised after 16 hours of incubation could be discerned, allowing for each plant to be classified as exhibiting low metabolism ($EMR_{L=}$ 100-80% of herbicide remaining), intermediate metabolism ($EMR_{I=}$ 79-50% of herbicide remaining), or resistant metabolism ($EMR_{R=}$ 49-0% of herbicide remaining). These three levels of EMR were used as they have been found by Bayer CropScience to correlate with agronomic problems within the field. Populations with low metabolism will pose no agronomic problem, populations with intermediate metabolism may pose a problem to the farmer, and populations with resistant metabolism will be an agronomic problem if population densities are great enough. To compare ALS and ACCase EMR, a weighted EMR index was calculated (Equation 2.1) for each mode of action to summarise the population's level of EMR. The index was designed so, for example, a population containing a mixture of metabolically resistant and susceptible individuals could be distinguished from a population of metabolically intermediate individuals. To calculate this index for each population, the number of plants (n) in each of the three metabolism categories was counted. The %_remaining (the percentage of herbicide remaining after 16 hours of

incubation) in each group was summed together, and multiplied by the number of plants in that group. The values for EMR_L, EMR_I, and EMR_R were summed together, before being divided by 6400 (which represents the total number of plants (n_{total}) multiplied by the maximum %_remaining of each plant (100) multiplied by n_{total} (Equation 2.1). The resulting value is then subtracted from 1, to get a value between 0 (all individuals are susceptible and cannot metabolise any herbicide) and 1 (all individuals are resistant and can metabolise all of the herbicide) that represents EMR.

$$1-\left(\frac{((n(EMR_L)*\Sigma(\%\_remaining(EMR_L)) + (n(EMR_I)*\Sigma(\%\_remaining(EMR_I)) + n(EMR_R)*\Sigma(\%\_remaining(EMR_R))}{6400}\right)$$

(Equation 2.1)

The correlation between ALS and ACCase EMR indices, divided into mesosulfuron-methyl + iodosulfuron-methyl-sodium treated and mesosulfuron-methyl + iodosulfuron-methyl-sodium untreated populations were evaluated using a generalized linear model in R (R Development Core Team 2012), incorporating mesosulfuron-methyl + iodosulfuron-methyl-sodium treated and mesosulfuron-methyl + iodosulfuron-methyl-sodium untreated populations as a factor.

2.3.5.5 Analysing the Genetic isolation of populations

To investigate the isolation of the ninety-two populations by distance, the five ACCase and two ALS SNPs studied were used with a method similar to that of Délye *et al* (2010). A genetic distance (F_{st}) matrix between all populations was computed using Weir & Cockerham F_{st} (Weir & Cockerham 1984) in the Geneland package of R (R Development Core Team 2012). The geographic distance (km) was also calculated between all fields to create a matrix. Using the values of the F_{st}

matrix and the geographic distance matrix, a linear regression model was then plotted between $\log_e$(geographic distance (km)) and $F_{st}/(1- F_{st})$ in R (R Development Core Team 2012) to determine their correlation. If a significant positive correlation exists between F_{st} and geographic distance (km), then this would indicate geographically close populations are genetically similar, hence interbreeding.

2.4 Results

2.4.1 Prevalence of phenotypic ALS and ACCase resistance in the UK

Of the ninety-two *A. myosuroides* populations sampled from across the UK in 2011, 69 (75%), 12 (13%), 11 (12%) exhibited a resistant, intermediate, and susceptible phenotype to a UK field dose of the ALS herbicide mesosulfuron-methyl (14g a.i ha^{-1}) + iodosulfuron-methyl-sodium (2.4g a.i ha^{-1}) respectively (Figure 2.2(a)). Of the ninety-two populations, 92, (100%), 0 (0%), 0 (0%) exhibited a resistant, intermediate, and susceptible phenotype to a UK field dose of the ACCase herbicide clodinofop-propargyl (60g a.i ha^{-1}) respectively (Figure 2.3(a)).

2.4.2 Relationship between ALS and ACCase phenotype and resistance mechanisms

Sixty-nine of the ninety-two *A. myosuroides* populations sampled from the UK in 2011 possessed at least one mechanism - target-site or enhanced metabolism - of ALS resistance. This is less than the 81 populations (69 phenotypically resistant + 12 phenotypically intermediate) exhibiting phenotypic resistance to mesosulfuron-methyl (14g a.i ha^{-1}) + iodosulfuron-methyl-sodium (2.4g a.i ha^{-1}) identified in section 2.4.1. The discrepancy most likely arises from all individuals used to identify ALS TSR and EMR in phenotypically resistant populations being susceptible by chance. Of the sixty-nine populations possessing a mechanism of ALS resistance,

twenty-four were resistant through enhanced metabolism only, and nine were resistant via target-site resistance only. When assessing the populations for multiple mechanisms of resistance, thirty-six populations had both mechanisms of resistance, target-site and enhanced metabolism (Figure 2.2(b)).

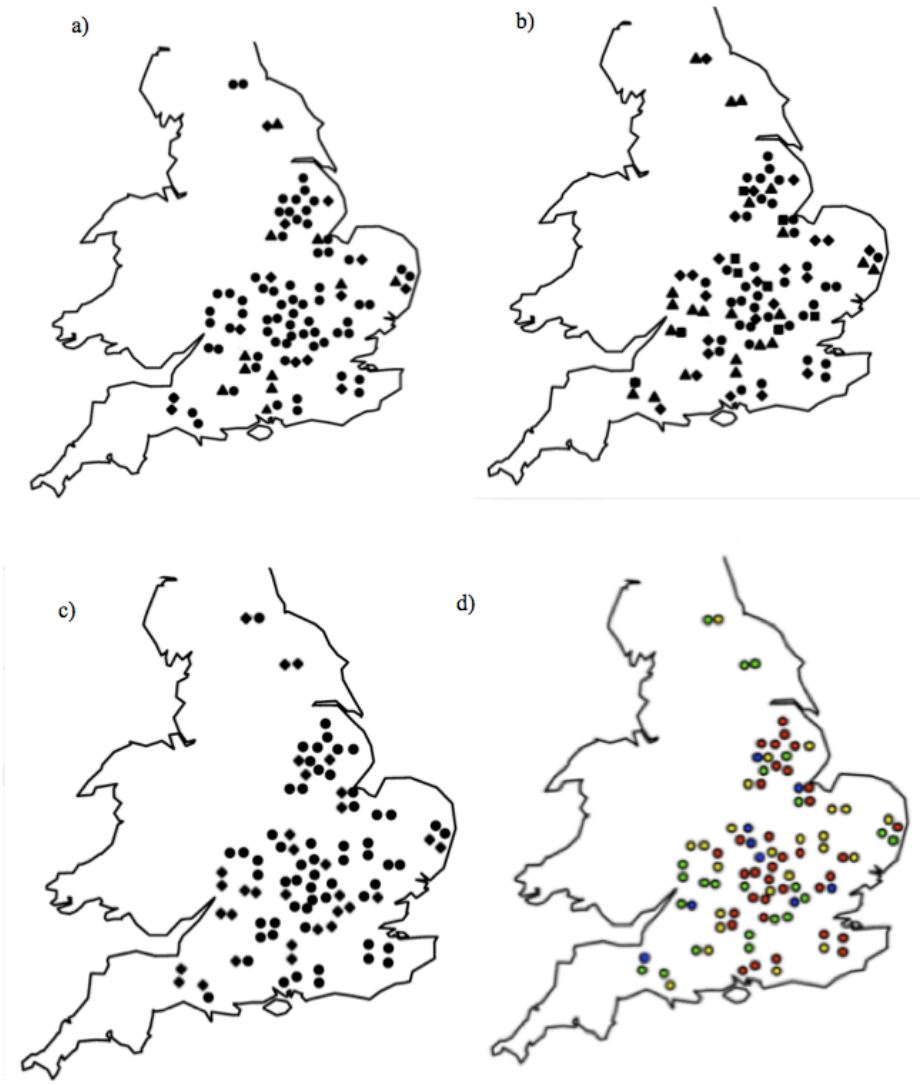


Figure 2.2: Maps of (a) ALS phenotypic resistance, (b) combinations of ALS resistance mechanism, (c) ALS EMR, and (d) ALS TSR in the UK in 2011. For ALS phenotypic resistance (*a*), resistant (circle), intermediate (diamond), and susceptible (triangle) phenotypes are indicated. For combinations of ALS resistance mechanism (*b*) populations with no mechanism of resistance (triangle), TSR only (square), EMR only (diamond), both TSR & EMR (circle) are shown. For ACCase EMR (*c*), EMR (circle), and no EMR (triangle) are indicated. For ALS TSR (*d*), populations identified with at least one individual possessing at least one Pro-197 (blue), Trp-574 (yellow), both Pro-197 & Trp-574 mutations (red), or wildtype (green) allele is indicated. Points are marked as near to the GPS location of the field as possible without obstructing any other point.

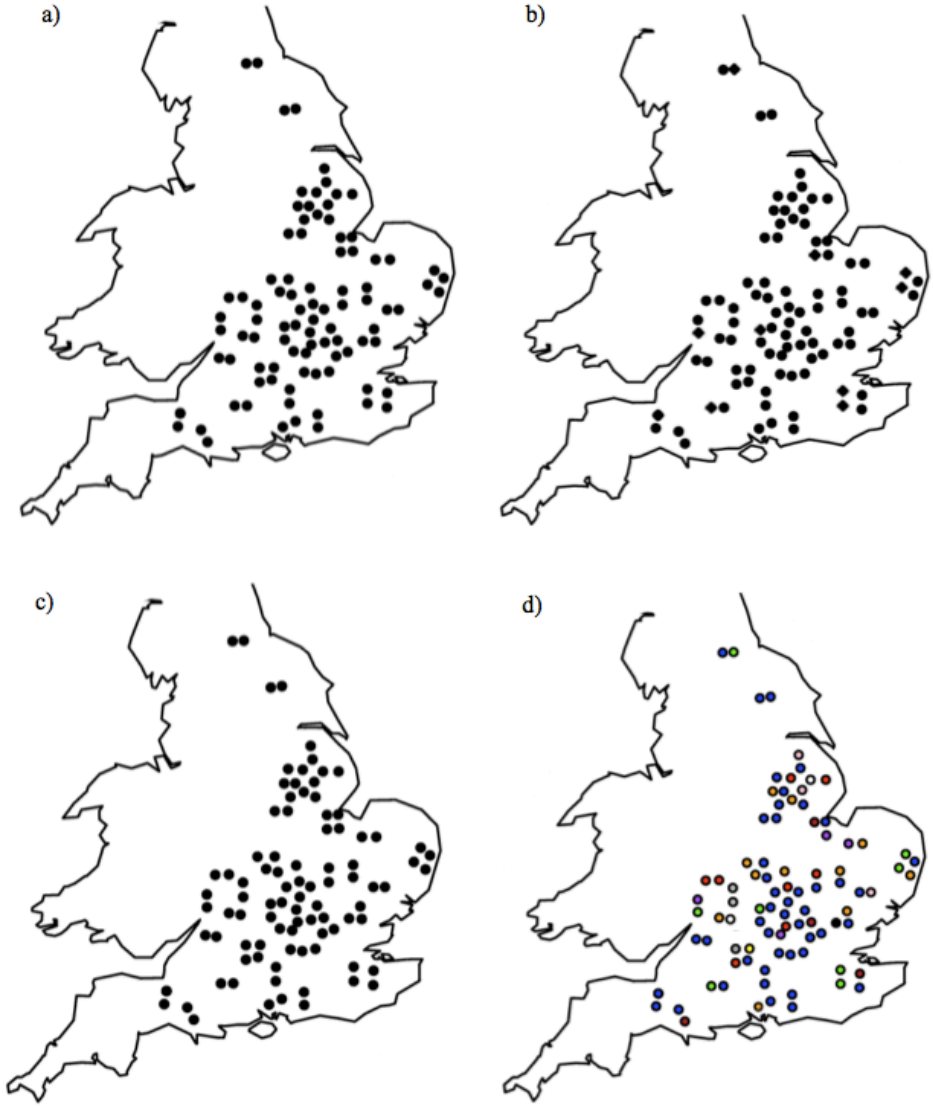


Figure 2.3: Maps of (a) ACCase phenotypic resistance (b) combinations of ACCase resistance mechanism, (c) ACCase EMR and (d) ACCase TSR in the UK in 2011. For ACCase phenotypic resistance (*a*), resistant (circle), intermediate (diamond), and susceptible (triangle) phenotypes are indicated. For combinations of ACCase resistance mechanism (*b*) populations with no mechanism of resistance (triangle), TSR only (square), EMR only (diamond), both TSR & EMR (circle) are shown. For ACCase EMR (*c*), EMR (circle), and no EMR (triangle) are indicated. For ACCase TSR (*d*), populations identified with at least one least one Ile-1781 (blue), Asp-2078 (yellow), Ile-1781+Asp-2078 (red), Ile-1781+Ile-2041 (orange), Ile-1781+Gly-2096 (pink), Ile-1781+Trp-2027 (brown), Ile-1781+Ile-2041+Asp-2078 (purple), Ile-1781+Ile-2041+Gly-2096 (white), Ile-1781+Trp-2027+Ile-2041 (grey), Ile-1781+Trp-2027+Asp-2078 (dark grey), Ile-1781+Trp-2027+Ile-2041+Gly-2096 (black) or wildtype (green) allele is indicated. Points are marked as near to the GPS location of the field as possible without obstructing any other point.

Seventeen of the thirty-six populations that expressed both mechanisms of resistance had Pro-197 mutations and enhanced metabolism, ten had Trp-574 mutations and enhanced metabolism, and nine populations had both Pro-197 mutations, Trp-574 mutations and enhanced metabolism. All of the 92 resistant populations that exhibited phenotypic resistance to a UK field dose of the ACCase herbicide clodinofop-propargyl (60g a.i ha^{-1}) also possessed an identifiable mechanism of resistance. All ninety-two populations exhibited ACCase enhanced metabolism, with eighty-two populations possessing target-site resistance as well (Figure 2.3(b)).

2.4.3 Prevalence of ALS and ACCase enhanced metabolism

A population was characterised as exhibiting mesosulfuron (ALS) or fenoxaprop-p-ethyl (ACCase) enhanced metabolism if more than two plants that could the herbicide by 20 - 50%, or more than one plant that could metabolise the herbicide by more than 50%. Sixty populations exhibited enhanced metabolism to mesosulfuron. Of the 736 plants tested for ALS enhanced metabolism, 269 (37%) expressed some degree of mesosulfuron metabolism (i.e. could metabolise more than 20% of the mesosulfuron applied) (Figure 2.2(c)). All ninety-two populations exhibited enhanced metabolism of fenoxaprop. Of the 736 plants tested, 639 (87%) expressed some degree of fenoxaprop metabolism (Figure 2.3(c)).

2.4.4 Prevalence of ALS target-site resistance

Forty-five of the ninety-two populations possessed at least one ALS target-site mutation (Table 2.4). Individuals from thirty-three and twenty-three populations possessed Pro-197 and Trp-574 mutations respectively; twenty-two populations possessed only Pro-197 target-site mutations, twelve populations had only Trp-574

mutations, and eleven populations had both Pro-197 and Trp-574 mutations (either in the same or different individuals) (Figure 2.2(d)).

Table 2.4 The number of Pro-197 and Trp-574 mutations identified within the 92 populations surveyed in 2011. The number of populations identified as having at least one individual with the specified ALS mutation is shown (along with this number as a percentage of the 92 populations tested). The total number of individuals that possessed each mutation is shown, including the number of heterozygous (RS) and homozygous (RR) individuals (along with this number as a percentage of the 736 individuals tested). The allele frequencies of each mutation across all populations and the total frequency of all ALS alleles are also shown.

ALS	Amino acid change	Total number of populations with each mutation (n=92)	Total number of plants with each mutation (n=736)	Number of RS plants for each mutation (n=736)	Number of RR plants for each mutation (n=736)	Allele Frequency
	Pro-197-Thr	32 (35%)	139 (19%)	139 (19%)	0 (0%)	0.0944
197	**Pro-197-Leu**	1 (1%)	1 (<1%)	1 (<1%)	0 (0%)	0.0007
	Pro-197- His	1 (1%)	5 (<1%)	5 (<1%)	0 (0%)	0.0033
574	**Trp-574-Leu**	23 (25%)	94 (13%)	51 (7%)	43 (6%)	0.0931
Total ALS mutations		45 (49%)	239 (32%)	196 (27%)	43 (6%)	0.3060

In total, 736 plants (eight plants from each of the 92 populations) were tested for ALS target-site resistance. One Trp-574 mutation (Trp-574-Leu) and three different Pro-197 mutations were identified: Pro-197-Thr, Pro-197-His, and Pro-197-Leu. Of the Pro-197 mutations identified, Pro-197-Thr mutations were in greatest frequency (Table 2.4). Plants with Pro-197 mutations were more frequently identified than those with Trp-574 mutations; however, all plants with Pro-197 mutations were heterozygous, compared with 6% of plants with Trp-574 mutations being homozygous (Table 2.3). The allele frequency of Pro-197-Thr mutations (0.0944 of 1,472 Pro-197 alleles) was similar to that of Trp-574-Leu (0.0931 of 1,472 Trp-574 alleles). In total, 30.60% of the 1,472 ALS alleles studied contained resistance-conferring mutations (Table 2.4).

Due to the small number of individuals sampled in each population, determining Hardy-Weinberg equilibrium within each of the populations possessing Pro-197

target-site mutations was not possible. However, across these populations mutations were found not to be in Hardy-Weinberg equilibrium. Pro-197 mutations were identified in 33 populations. Of the 264 plants with Pro-197 target-site mutations, 119 were wildtype, 145 were heterozygous and 0 were homozygous. This gives a wildtype: heterozygous: homozygous ratio of 1: 1.8: 0, significantly different (Fisher's exact test, $P < 0.05$) from the 1: 2: 1 ratio expected if at Hardy-Weinberg equilibrium. The observed ratio is not significantly different (Fisher's exact test, $P > 0.05$) to the ratio of 1: 2: 0 observed when the homozygous are recessive lethal alleles.

Trp-574 mutations were also not in Hardy-Weinberg equilibrium. Trp-574 mutations were identified in 23 populations. Of the 184 plants with Trp-574 mutations, 90 were wildtype, 51 were heterozygous, and 43 were homozygous. This gives a wildtype: heterozygous: homozygous ratio of approximately 2: 1: 1, a ratio significantly different (Fisher's exact test, $P < 0.05$) from the 1: 2: 1 ratio expected if at Hardy-Weinberg equilibrium.

2.4.5 Prevalence of ACCase target-site resistance

All of the ninety-two *A. myosuroides* populations sampled from the UK in 2011 possessed a mechanism of ACCase resistance, target-site and/or enhanced metabolism. Of the ninety-two ACCase resistant populations, eighty-two possessed at least one ACCase target-site mutation at one of the five positions in the ACCase gene: Ile-1781, Trp-2027, Ile-2041, Asp-2078, or Gly-2096 (Table 2.5). Populations with only Ile-1781 mutations were found in greatest frequency, with all other

ACCase mutations co-occurring in populations with Ile-1781 mutations, except one population that possessed Asp-2078 mutations only (Table 2.5).

Table 2.5: Combination of ACCase target-site mutations identified in the 92 UK populations studied in 2011. The number of populations with each mutation combination is represented.

Mutation combination	Number of Populations
Ile-1781	46
Ile-1781, Ile-2041	11
Ile-1781, Asp-2078	7
Ile-1781, Ile-2041, Asp-2078	4
Ile-1781, Trp-2027	4
Ile-1781, Gly-2096	3
Ile-1781, Trp-2027, Ile-2041	2
Ile-1781, Ile-2041, Gly-2096	2
Asp-2078	1
Ile-1781, Trp-2027, Asp-2078	1
Ile-1781, Trp-2027, Ile-2041, Gly-2096	1
Total	82

From the 736 plants tested, three different amino acid changes at position Ile-1781 (Ile-1781-Leu, Ile-1781-Thr, Ile-1781-Val), and one amino acid change at positions Trp-2027 mutation (Trp-2027-Cys), Ile-2041 (Ile-2041-Asn), Asp-2078 (Asp-2078-Gly) and Gly-2096 (Gly-2096-Ala) were identified. Ile-1781-Leu mutations were found in the greatest frequency (Table 2.6).

In total, 68.89% of the 1,472 alleles studied were ACCase resistance conferring mutations (Table 2.6). Due to the small number of individuals sampled in each population, determining Hardy-Weinberg equilibrium individually within the mutation containing populations was not possible. However, across these populations all of the ACCase mutations were in a ratio significantly different (Fisher's exact test, $P < 0.05$) to the 1: 2: 1 ratio expected if at Hardy-Weinberg equilibrium (Table 2.7).

Table 2.6: The number of Ile-1781, Trp-2027, Ile-2041, Asp-2078, and Gly-2096 mutations identified within the 92 populations surveyed in 2011. The number of populations identified as having at least one individual with the specified ACCase mutation is shown (along with this number as a percentage of the 92 populations tested). The total number of individuals that possessed each mutation is shown, including the number of heterozygous (RS) and homozygous (RR) individuals (along with this number as a percentage of the 736 individuals tested). The allele frequencies of each mutation across all populations and the total frequency of all ACCase alleles are also shown.

ACCase	Amino acid change	Total number of populations with each mutation (n=92)	Total number of plants with each mutation (n=736)	Number of RS plants for each mutation (n=736)	Number of RR plants for each mutation (n=736)	Allele Frequency
	Ile-1781-Leu	73 (79%)	452 (61%)	347 (47%)	105 (14%)	0.3784
1781	**Ile-1781-Thr**	8 (9%)	15 (2%)	15 (2%)	0 (0%)	0.0102
	Ile-1781-Val	3 (3%)	6 (1%)	2 (<1%)	4 (<1%)	0.0068
2027	**Trp-2027-Cys**	9 (10%)	14 (2%)	14 (2%)	0 (0%)	0.0095
2041	**Ile-2041-Asn**	20 (22%)	46 (6%)	44 (6%)	2 (<1%)	0.0326
2078	**Asp-2078-Gly**	14 (15%)	29 (4%)	27 (4%)	2 (<1%)	0.0211
2096	**Gly-2096-Ala**	6 (7%)	16 (2%)	16 (2%)	0 (0%)	0.0109
Total		82 (89%)	578 (79%)	465 (63%)	113 (15%)	0.6889

Table 2.7: Hardy-Weinberg proportions for Ile-1781, Trp-2027, Ile-2041, Asp-2078, and Gly-2096 ACCase target-site mutations.

ACCase	Total number of populations with each mutation	Number of SS plants with each mutation in the Total number of populations with each mutation	Number of RS plants with each mutation in the Total number of populations with each mutation	Number of RR plants with each mutation in the Total number of populations with each mutation	Ratio
Ile-1781	73	132	364	109	1: 3: 1
Trp-2027	9	58	14	0	4: 1: 0
Ile-2041	20	114	44	2	57: 22: 1
Asp-2078	14	83	27	2	42: 14: 1
Gly-2096	6	32	16	0	2: 1: 0

2.4.6 Genetic isolation of populations

To investigate the isolation of the ninety-two populations by distance, the five ACCase and two ALS studied were used with a method similar to that of Délye *et al* (2010). A genetic distance (F_{st}) matrix (Weir & Cockerham 1984) and geographic distance (km) matrix was assessed against each other using a linear regression model ($\log_e$(geographic distance (km)) against $F_{st}/(1- F_{st})$). If a significant positive correlation exists between F_{st} and geographic distance (km), then this would indicate geographically close populations are genetically similar, hence interbreeding. There was no correlation between Fst and geographic distances when evaluated using a

linear model for the ninety-two UK populations studied (adjusted $r^2 = 0.0585$, F = 1.192, P = $7.031e^{-05}$), inferring that populations that are geographically close to one another (or further apart for that matter) are not interbreeding. This result is similar to significant non-correlation observed from UK populations by Délye *et al* (2010) (F = 48.5, P = $3.86*10^{-12}$, adjusted $r^2 = 0.013$).

2.4.7 Effect of herbicide treatment on resistance identification

Of the *A. myosuroides* populations sampled from UK farms that were experiencing control issues, those that had been treated with mesosulfuron-methyl + iodosulfuron-methyl-sodium in the year of sampling (2011) exhibited a significantly (Table 2.8) higher frequency of resistance than those that had not. There was a significant correlation between the occurrence of ALS and ACCase resistant populations in mesosulfuron-methyl + iodosulfuron-methyl-sodium treated samples, but no significant correlation between the occurrence of ALS and ACCase resistant populations in mesosulfuron-methyl + iodosulfuron-methyl-sodium untreated samples due to the lower frequencies of ALS resistant populations identified.

Table 2.8: Differences between Atlantis treated and untreated populations and correlations between ALS and ACCase modes of action. The levels of susceptible, intermediate and resistant populations for mesosulfuron-methyl + iodosulfuron-methyl-sodium treated and untreated populations are shown for ALS and ACCase MOA. Significant differences (P < 0.05) with a Fisher's exact test are indicated with an asterisk (*).

Populations		Number of populations resistant to ALS MOA	Number of populations resistant to ACCase MOA	ALS vs. ACCase P-Value	Treated vs. Untreated P-Value
Treated	Susceptible	2	0	0.495	0.000*
	Intermediate	3	0	0.242	
	Resistant	41	46	0.557	
Untreated	Susceptible	9	0	0.003*	
	Intermediate	10	0	0.001*	
	Resistant	27	46	0.000*	

As mentioned in section 2.4.2, there was identified a discrepancy between the level of ALS phenotypic resistance and ALS resistance mechanisms, most likely caused by all individuals used to identify ALS TSR and EMR in phenotypically resistant populations being susceptible by chance. Of the mesosulfuron-methyl + iodosulfuron-methyl-sodium treated populations, four (9%) were resistant as a result of target-site resistance only, ten (22%) were resistant as a result of only enhanced metabolism, and twenty-six (57%) had both mechanisms of resistance. Of the mesosulfuron-methyl + iodosulfuron-methyl-sodium untreated populations, four (9%) were resistance as a result of target-site resistance only, fourteen (30%) were resistance as a result of only enhanced metabolism, and ten (22%) had both mechanisms of resistance. The only significant difference (Fisher's exact test, $P < 0.05$) between the occurrence of ALS resistance mechanisms in mesosulfuron-methyl + iodosulfuron-methyl-sodium treated and mesosulfuron-methyl + iodosulfuron-methyl-sodium untreated populations was found between the number of populations with both mechanisms of resistance.

2.4.8 Correlation between the extent ALS and ACCase target-site resistance

Eight plants for each population were tested for the presence of ALS (Pro-197 and Trp-574) and ACCase (Ile-1781, Trp-2027, Ile-2041 Asp-2078 and Gly-2096) target-site mutations. For each population the proportion of plants possessing at least one ALS mutation was calculated, as well as the proportion of plants possessing at least one ACCase mutation. To assess whether there was a correlation between the frequency of ALS and ACCase TSR in mesosulfuron-methyl + iodosulfuron-methyl-sodium treated and mesosulfuron-methyl + iodosulfuron-methyl-sodium untreated populations the proportion of ALS and ACCase TSR was compared using a

generalized linear model (Figure 2.4). No significant correlation (P = 0.9771) was identified between ALS and ACCase target-site resistance for either mesosulfuron-methyl + iodosulfuron-methyl-sodium treated and mesosulfuron-methyl + iodosulfuron-methyl-sodium untreated populations (odds ratio ("slope") = 1.02) (Figure 2.4). However with an intercept of 0.38 and 0.15 for mesosulfuron-methyl + iodosulfuron-methyl-sodium treated and mesosulfuron-methyl + iodosulfuron-methyl-sodium untreated populations respectively, mesosulfuron-methyl + iodosulfuron-methyl-sodium untreated populations exhibited a significantly (P = 0.003) lower proportion of ALS target-site resistance than mesosulfuron-methyl + iodosulfuron-methyl-sodium untreated populations (Figure 2.4).

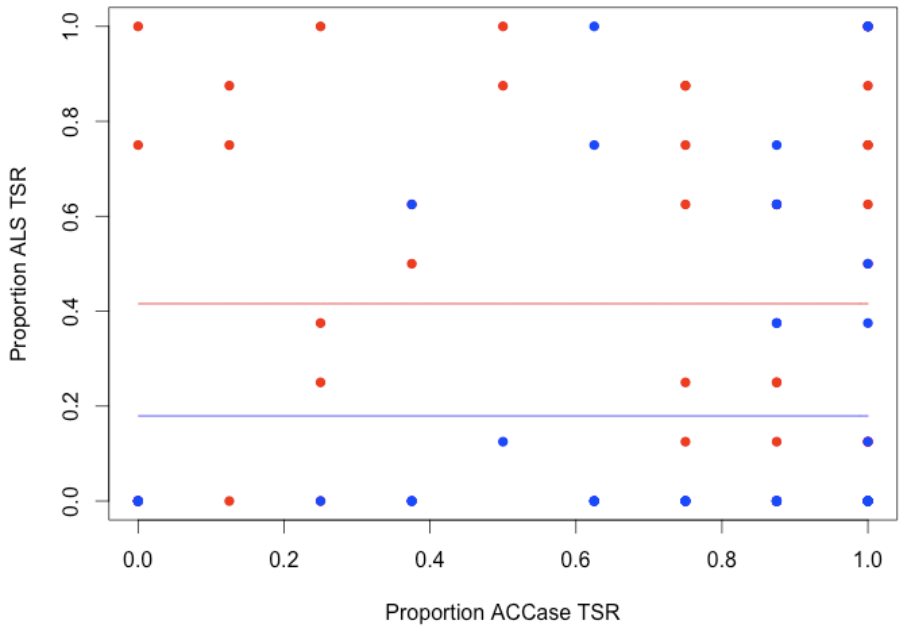


Figure 2.4: Correlation between ACCase and ALS TSR proportions for mesosulfuron-methyl + iodosulfuron-methyl-sodium treated (red) and mesosulfuron-methyl + iodosulfuron-methyl-sodium untreated (blue) populations.

2.4.9 Correlation between the extent ALS and ACCase enhanced metabolism

For each of the ninety-two UK *A. myosuroides* populations sampled in 2011, the ALS and ACCase EMR index was calculated (as described in section 2.3.5.4) and a

generalized linear model plotted between the values to identify if a significant correlation exists between them, i.e. do populations with higher levels of ACCase EMR possess higher levels of ALS EMR. The populations were split into mesosulfuron-methyl + iodosulfuron-methyl-sodium treated and mesosulfuron-methyl + iodosulfuron-methyl-sodium untreated populations to assess whether herbicide treatment had an effect on the level of EMR identified (Figure 2.4). Populations treated with mesosulfuron-methyl + iodosulfuron-methyl-sodium exhibited no significant (P = 0.6419) relationship between ACCase EMR and ALS EMR, with an odds ratio ("slope") of 0.9937 and an intercept of 0.63 (Figure 2.4). The odds ratio of populations untreated with mesosulfuron-methyl + iodosulfuron-methyl-sodium exhibited no significant (P = 0.0715) relationship between ACCase EMR and ALS EMR, with an odds ratio ("slope") of 1.4777 and an intercept in 0.0374 (Figure 2.4).

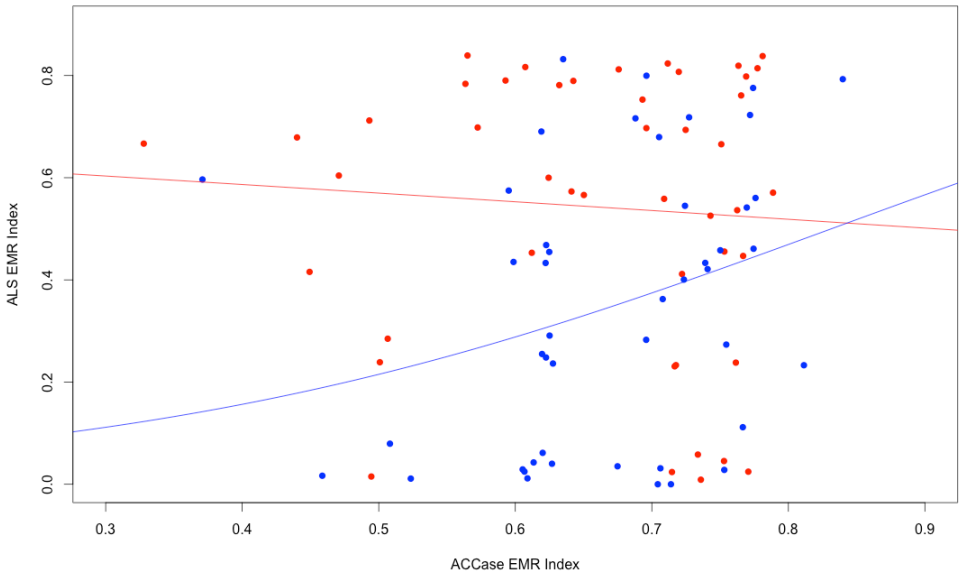


Figure 2.5: Correlation between ACCase and ALS EMR indexes for mesosulfuron-methyl + iodosulfuron-methyl-sodium treated (red) and mesosulfuron-methyl + iodosulfuron-methyl-sodium untreated (blue) populations.

2.5 Discussion

2.5.1 ALS frequency of resistance and resistance mechanisms

Eighty-one of the ninety-two *A. myosuroides* populations studied exhibited resistance – either a resistant (69) or intermediate resistant (12) phenotype - to the UK recommended field rate of the ALS herbicide mesosulfuron-methyl + iodosulfuron-methyl-sodium. Even though ALS resistance was identified at a high frequency within the ninety-two *A. myosuroides* populations studied in 2011, the infestation level within the fields may not yet be an agronomic problem for the farmer. Therefore, the results from this survey can be used as early diagnostic information by the farmer, so they have the opportunity to react with better integrated weed management (IWM) before the resistance problem becomes an agronomic issue.

The high level of ALS resistance identified in the ninety-two UK *A. myosuroides* here is similar to other *A. myosuroides* surveys and other weed species of other countries. In the Côte d'or region of France, 121/124 (98%) *A. myosuroides* populations sampled exhibited resistance to flupyrsulfuron, with 50% of all plants tested being resistant (Chauvel *et al* 2006). In Germany, Petersen (2011) identified resistance to the ALS herbicides in approximately 6% of 236 populations tested, While sixteen percent (17/104) of populations from three regions of Germany sampled in 2010 exhibited resistance to mesosulfuron + iodosulfuron (Hess *et al* 2012). In the UK, 75% of 122 non-randomly sampled populations exhibited resistance to mesosulfuron-methyl + iodosulfuron-methyl-sodium (Hull *et al* 2014). Likewise, 98% (354/362) of *Lolium rigidum* populations sampled from Australia in 2010 expressed resistance to the ALS herbicide sulfometuron (Owen *et al* 2014).

A mixture of mechanisms was found to underlie resistance at the population level – just target-site resistance, just enhanced metabolism, and the co-occurrence of both mechanisms. At least one Pro-197 ALS target-site mutation was identified in 37% of the 92 UK *A. myosuroides* populations sampled populations, with 20% of plants 736 plants studied exhibiting the mutation; both of these frequencies are higher than the 25% of populations and 19% of plants respectively observed with Trp-574 target-site mutations. The number of populations identified with at least one Pro-197 target-site mutation was higher than the total of 20% (21/104) populations from three regions of Germany in 2010, while the number of populations identified with at least one Trp-574 target-site mutation was comparable - 23% (23/104) of populations – to this study (Hess *et al* 2012). The frequencies of plants possessing Pro-197-Thr and Trp-574-Leu target-site mutations identified here are higher than the 7% of 570 plants from 19 random populations sampled between 2009 and 2011 expressing Pro-197-Thr mutations and 7% expressing Trp-574-Leu mutations (Moss *et al* 2014). One unexpected result from the survey was a lack of homozygous Pro-197-Thr mutations, being potentially indicative of recessive lethality associated with homozygous Pro-197-Thr mutations that may have an effect on how this mutation is managed. This finding has been alluded to previously during the characterisation of ALS resistant *A. myosuroides* in the UK, Marshall and Moss (2008) noted that all individuals (of 42 mesosulfuron-methyl + iodosulfuron-methyl-sodium resistant samples taken from seven populations) were heterozygous for the Pro-197-Thr mutation. This identification of only heterozygous Pro-197-Thr mutations here and by Marshall and Moss (2008) is contradicted by studies identifying homozygous Pro-197-Thr mutations in populations of *Lolium rigidum* (Collavo and Sattin 2014), *Papaver rhoeas* (Délye *et al* 2011a) and *Alopecurus myosuroides* (Marshall *et al* 2013).

Enhanced metabolism was identified in greater prevalence than seen in other UK studies. Previously, 20% of 570 plants from 19 random populations sampled between 2009 and 2011 survived treatment with an ALS herbicide through a mechanism of non-target-site resistance (Moss *et al* 2014). The figure quoted by Moss *et al* (2014) is an estimate based on the number of phenotypically ALS resistant individuals that did not possess target-site resistance. Therefore, the 37% of plants identified using HPLC metabolite analysis will be a more accurate estimate of the proportion of individuals that express ALS enhanced metabolism in UK populations of *A. myosuroides.* More importantly, assessing enhanced metabolism with HPLC has allowed for the occurrence of target-site and enhanced metabolism within the same population to formally identified - 39% of populations - for the first time in UK populations of *A. myosuroides*. Resistance to ALS modes of action was identified as being lower than that of ACCase resistance: this is most likely as a result of ALS inhibitors being used for a much shorter period of time.

2.5.2 ACCase frequency of resistance and resistance mechanisms

All of the ninety-two UK populations sampled contained plants that were phenotypically resistant to the ACCase herbicide clodinofop-propargyl, concurring with previous studies that resistance to herbicide with ACCase modes of action is throughout *A. myosuroides'* main distribution (Moss *et al* 2007; Délye *et al* 2007; Délye *et al* 2010; Hess *et al* 2012; Hull *et al* 2014; Keshtkar *et al* 2015). This high level of ACCase phenotypic resistance is similar to surveys of other species: 90% (165/270) of *L. rigidum* populations sampled from Australia (1998, 2003, 2008) expressed resistance to dicolfop-methyl (Malone *et al* 2013), while 96% of

Australian *L. rigidum* populations from 2010 exhibited dicolfop-methyl resistance (Owen *et al* 2014).

The majority of the populations contained target-site mutations. Again, this result is in line with other studies of ACCase resistance from across northern Europe (Moss and Perryman 2007; Délye *et al* 2007; Délye *et al* 2010; Hess *et al* 2012; Moss *et al* 2014). Ile-1781 mutations were identified in the greatest frequency - similar to other surveys e.g. Délye *et al* (2010) - with the mutation being present in all except one of the populations that exhibited ACCase target-site resistance. Ile-1781 mutations being the most frequent in *A. myosuroides* is in contrast to a survey of *L. rigidum* in Australia conducted by Malone *et al* (2013), in which Asp-2041 mutations were most frequently identified ACCase target-site mutation. This most likely because the ACCase inhibitor applied can have an effect on the mutation selected (e.g. Asp-2078-Gly mutations confer high-level resistance to (and are therefore selected by) many cyclohexanedione (CHD) ACCase inhibitors, except the CHD clethodim which only confers low-level CHD resistance (Powles and Yu 2010)).

All populations possessed the ability to metabolise the ACCase herbicide fenoxaprop to some degree. During the phenotypic analysis of these populations, the ACCase herbicide clodinofop-propargyl was used to identify ACCase resistant populations. However, enhanced metabolism was identified using fenoxaprop, as it is assumes that metabolic resistance to fenoxaprop and clodinofop-propargyl are linked (although this might not be the case in all the populations). With all populations expressing phenotypic resistance to clodinofop-propargyl and all populations

exhibiting fenoxaprop enhanced metabolism, clodinofop-propargyl and fenoxaprop does appear to be closely linked within the *A. myosuroides* populations studied.

With all populations and eighty-seven percent of plants able to metabolise fenoxaprop, these finding agrees with the study of Délye *et al* (2007), which predicted that 75% of all ACCase resistant individuals have enhanced metabolism. As with ALS enhanced metabolism, ACCase metabolism was identified using HPLC metabolite analysis to obtain a more accurate estimate of the proportion of individuals that express ACCase enhanced metabolism in UK populations of *A. myosuroides.* Due to the high incidence of ACCase target-site resistance and ACCase enhanced metabolism, the number of populations possessing both mechanisms of resistance was high (82/92 populations). The identification and prevalence of target-site and enhanced metabolic resistance together within a single population may be an indication of the importance of both mechanisms in the evolution of resistance at the population level. It is also possible that both mechanisms developed independently in individuals of that population, but with time and the out-crossing, both mechanisms appeared together when the resistance had significant time to be established.

2.5.3 Correlations between ALS and ACCase resistance mechanisms

When comparing whether there is any significant correlation between the occurrence of ALS and ACCase resistance, it was found that there is a significantly (Fisher's exact test, $P < 0.05$) lower number of populations with ALS resistance than ACCase resistance. This is an indication that the two resistances are not linked, as a population with ACCase resistance does not always exhibit ALS resistance. There was also no significant correlation identified between ALS and ACCase target-site

resistance when analysed with a generalized linear model, again, indicating that ALS and ACCase TSR are independently evolved mechanisms of resistance.

Conversely, in populations not treated with mesosulfuron-methyl + iodosulfuron-methyl-sodium in the year of sampling, there was a positive relationship – albeit non-significant (P = 0.007) between the indexes of ALS EMR and ACCase EMR, with populations exhibiting higher ACCase EMR indexes also displaying larger ALS EMR indices in populations untreated with mesosulfuron-methyl + iodosulfuron-methyl-sodium in the year of sampling. This relationship was not seen in populations treated with mesosulfuron-methyl + iodosulfuron-methyl-sodium. It has been suggested that alleles conferring enhanced metabolism to aryloxyphenoxypropionate ACCase inhibitors may also confer a degree of "pre-adaptation" to ALS modes of action (Délye *et al* 2011b). The relationship between ACCase and ALS EMR indices indicated here might be an indication of this. With mesosulfuron-methyl + iodosulfuron-methyl-sodium treated populations not displaying this affect, there is the implication that sampling populations selected with ALS herbicide in the year of collection - with ALS treated populations overestimating the frequency of resistance observed as discussed below - potentially inhibits the ability to identify important correlations between mechanisms and MOA.

Using the method of Délye *et al* (2010), the genetic isolation (calculated using the five ACCase and two ALS mutations) of the ninety-two populations by distance was calculated to identify whether the 92 populations surveyed are independently evolving. With no significant geographic patterns in the evolution of target-site resistance, it is implied that resistance is evolving independently in each population;

similar conclusion was found for UK populations studied by Délye *et al* (2010). However, the frequency of these SNPs in each population might be biased because of the herbicide treatment applied to the population in the year of sampling. To get a true indication of population independence, the results presented here will have to be compared with results of neutral SNP markers not related to genes selected by the herbicide from the same populations.

2.5.4 Effect of herbicide treatment and sampling on resistance identification

There was a difference in the frequency of ALS resistant *A. myosuroides* populations sampled from mesosulfuron-methyl + iodosulfuron-methyl-sodium treated and mesosulfuron-methyl + iodosulfuron-methyl-sodium untreated fields in 2011. Populations treated with mesosulfuron-methyl + iodosulfuron-methyl-sodium in the year of sampling exhibited higher levels of resistance, as these samples only represented resistant individuals that survived herbicide application, whereas the mesosulfuron-methyl + iodosulfuron-methyl-sodium untreated populations represented the whole population: susceptible and resistant individuals. Herbicide treatment in the year of seed collection removes the majority of emerged susceptible individuals from the population, leading to an overestimate of resistance frequency at the population level as estimates of resistance frequency are based on seeds produced by surviving (and therefore predominantly resistant) individuals and do not take account of the frequency of susceptible individuals within the seed bank. Sampling populations that are not treated with ALS herbicides in the year of seed collection is able to give a more accurate prediction of the frequency of ALS resistance within the entire population. This is important when discerning the presence and frequency of resistance within *A. myosuroides* populations. The herbicide that a population has

been treated with should be taken into account when sampling, and incorporated into the results and the conclusions that should be drawn from them. This enables a better idea of the level of resistance within a field, area, county or country to be established, and better management regimes to be produced based on this information.

As seed samples were collected in this study, the level of resistance in the population is not what is being assessed, but the frequency of resistance in the seed in the year of collection. This information is still important and informative to the farmer, as good *A. myosuroides* control may have been achieved with the herbicide (i.e. no visible *A. myosuroides* infestation in the field), but the results of the resistance tests presented here may indicate high levels of resistance. The results need to be placed in context. For this, information about the number and densities of survivors from which seed was collected is required. If the number of individuals surviving herbicide application was small, then the resistance problem may not be great at the time of collection. If there were a large number of individuals surviving herbicide treatment, there will be a large return of resistant seed, so there will be a greater resistance problem when the herbicide is applied subsequently, regardless of actual population frequency within the seedbank. From a management perspective therefore, high levels of resistance in seed collected are indicative of an actual or impending resistance problem, depending on infestation level within the field.

2.6 Conclusions

All ninety-two populations studied from an important arable area of the UK exhibited phenotypic resistance to an ACCase MOA, and eighty-one exhibited phenotypic resistance to an ALS MOA. From the perspective of weed management,

high levels of resistance to these essential MOA coupled with increased legislation and a lack of novel modes of action being identified, the ability to effectively manage *A. myosuroides* chemically in the UK is diminished. This puts greater emphasis on the use of integrated weed management. Furthermore, the high levels of target-site resistance and enhanced metabolism were identified for both MOA. The occurrence of enhanced metabolism could further exacerbate control issues as a result of cross-resistance to other chemical classes, further reducing options for chemical resistance management. The identification and prevalence of target-site and enhanced metabolic resistance together within a single population may be an indication of the importance of each mechanism in the evolution of resistance at the population level.

3.0 Monitoring the frequency of ACCase and ALS herbicide resistance and resistance mechanisms in UK populations of *Alopecurus myosuroides.*

3.1 Introduction

3.1.1 Epidemiological studies

Epidemiology can be defined as the study of the distribution and causes of conditions detrimental to human health, and the application of study information to cure these conditions (Last 2001). Understanding the epidemiology of diseases such as cholera (Kaper *et al* 1995), malaria (Greenwood 1997), HIV (Kilmarx 2009), and tuberculosis (Raviglione *et al* 1995), has contributed to their declining mortality rates. Epidemiological methods can be applied outside of the field of human health, and as such, are proving to be an essential tool when investigating the distribution and underling causes of antibiotic, insecticide, fungicide and herbicide resistance evolution (Zhan et al 2008; Hawkey and Jones 2009; Koella *et al* 2009; Pfaller 2012; Evans *et al* 2015).

3.1.2 Epidemiological studies of herbicide resistance evolution

At present, a large proportion of studies into herbicide resistance are "reactive"; that is they observe the outcome of selection and determine the proximate causes - physiological mechanisms that endow the evolution of resistance (Neve *et al* 2014). More proactive studies that observe populations during selection and the ultimate ecological and evolutionary factors that underpin resistance are required (Neve *et al* 2014).

The evolution herbicide resistance occurs over large temporal and spatial scales, making detailed long-term studies into resistance evolution impractical to conduct (Renton *et al* 2014). Therefore, small-scale experiments and simulation models can be used to identify the ultimate ecological and evolutionary factors that underpin resistance. For example, the model organism *Chlamydomonas reinhardtii,* a single-celled alga, has been used to investigate the effect that herbicide cycling and herbicide mixtures have on the evolution of herbicide resistance (Lagator *et al* 2013a,b). Similarly, simulation models have been used to establish the effect that herbicide mixtures (Diggle *et al* 2003), reduced herbicide application rates (Renton *et al* 2011), environmental heterogeneity of herbicide application (Richter *et al* 2002), and spatio-temporal dynamics of herbicide use (Jacquemin *et al* 2009; Richter *et al* 2002) have on the evolution of resistance.

Small-scale experiments and simulation models are useful tools in trying to elucidate the ultimate causes of resistance evolution and spread. However, studies that embrace an epidemiological approach are needed in order to confirm the findings of these experiments and models. To date, only one published study has adopted an epidemiological approach to explore the evolution of herbicide resistance. Evans *et al* (2015) studied the frequency of glyphosate resistant *Amaranthus tuberculatus* in 105 fields from Illinois (USA) against soil, landscape, and historical farm management data to identify factors associated with resistance evolution. Populations with the greatest frequencies of glyphosate resistant *A. tuberculatus* were associated with frequent glyphosate applications, high glyphosate doses, and lack of mode of action diversity within a cropping year (Evans *et al* 2015).

3.1.3 A. myosuroides herbicide resistance studies

As evident from the results presented in chapter two and numerous other studies (Chauvel *et al* 2006; Moss *et al* 2007; Délye *et al* 2007; Délye *et al* 2010; Petersen 2011; Hess *et al* 2012; Hull *et al* 2014; Keshtkar *et al* 2015), target-site and enhanced metabolic resistance to ALS and ACCase post-emergent modes of action are widespread. However these studies, including that reported in chapter 2, are reactive. Epidemiological studies that monitor the change in resistance over a number of generations in relation to management are needed. Through a combination of small-scale experiments, simulation modelling, and long-term epidemiological studies, a greater understanding of the temporal evolution of *A. myosuroides* herbicide resistance in relation to management can be distinguished, so that more effective *A. myosuroides* resistance control strategies can be developed.

3.2 Objectives

Work reported in this chapter aims to determine how the frequency of ALS and ACCase herbicide resistance, and the mechanisms that endow resistance, change over a four-year period between 2011 and 2014. Seventeen populations from a survey conducted in 2011, chosen for their contrasting frequencies of phenotypic resistance, and target-site and enhanced metabolism mechanisms to ALS and ACCase MOA, were re-sampled in 2012, 2013, and 2014. The frequencies of phenotypic resistance, target-site resistance and enhanced metabolism in each population/year were estimated, to describe how ALS and ACCase have evolved in these *A. myosuroides* populations over this period. This chapter is purely descriptive of the populations studied. Subsequent epidemiological analysis (Chapter 4) will determine how the frequency of ALS and ACCase resistance changes in relation to

weed management.

3.3 Materials and methods

3.3.1 Population sampling

To collect an annual sample of *A. myosuroides* seed from the seventeen fields, each field was visited at the end of July/beginning of August in 2012, 2013 and 2014 when *A. myosuroides* seed was mature (Figure 3.1). A sample was collected from all fields except those in which *A. myosuroides* was difficult to collect (e.g. certain, but not all fields sown with Oilseed rape (*Brassica napus*)). To ensure samples were random and representative of each *A. myosuroides* population, all tramlines in each field were walked for the entire field. Seed was uniformly sampled by collecting one seed-head from plants at 10 metre intervals: a maximum of 500 seed-heads were collected per field. A bulk seed sample from each field was collected in paper bags, and bags were stored in a drying room (relative humidity = 15%) until use. At each annual sampling, the presence of *A. myosuroides* across the field was scored on a scale of 0-5: 0 = no black-grass found, 1 = a low density of occasional solitary plants, 2 = occasional small patches, 3 = large patches, 4 = widespread throughout the field, 5 = a dense and serious infestation.

3.3.2 Collection of weed management data

Information about the weed management applied to each of the seventeen fields was collected for an eight-year period between 2007 and 2014 (farmers for 4 fields failed to provide management information). The information collected included details of all crops sown and their sowing dates, the product names, dates and doses of all herbicides applied (glyphosate, pre-emergent and post-emergent herbicide) during

this period, and details of soil cultivation and any other cultural weed control practices during each year. This information was collected through a combination of farm visits, telephone and email conversations with farmers, and provided in the form of either photocopied written records or printed computer records.

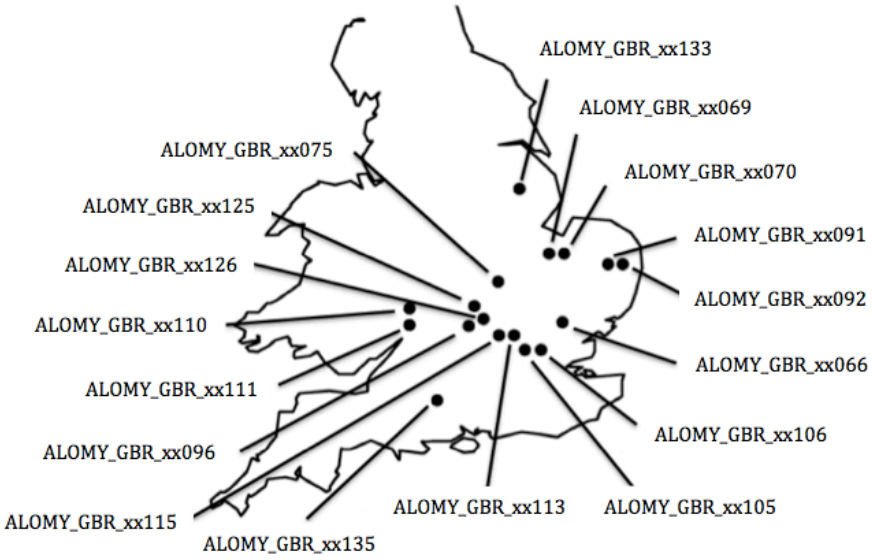


Figure 3.1: A map of the 17 *A. myosuroides* populations sampled between 2011 and 2014. Points are positioned as close to the GPS co-ordinates of the field without obscuring any other. Populations are labeled as ALOMY_GBR_xx092, for example. For each sampling year - 2012, 2013, 2014 – the xx in each label is replaced by the year sampled, so the 2012 sample of ALOMY_GBR_xx092 becomes ALOMY_GBR_12092. This method of labeling is used throughout.

3.3.3 Estimating phenotypic resistance frequency

After annual sampling, each seed population was phenotyped to estimate the proportion of individuals resistant to three doses of ALS and ACCase herbicide (0.5x 1x and 2x of the UK recommended field dose (1x) of each; a 0x control was also included for each MOA). The herbicides used were the ALS inhibitor mesosulfuron-methyl + iodosulfuron-methyl-sodium (0.5x = 6 + 1.2 g.ai. ha^{-1}, 1x = 12 + 2.4 g.ai. ha^{-1}, 2x = 24 + 4.8 g.ai. ha^{-1}) with the adjuvant Biopower (0.27 kg a.i ha^{-1}), and the ACCase inhibitor fenoxaprop-p-ethyl (0.5x = 41.5 g.ai ha^{-1}, 1x = 83 g.ai ha^{-1}, 2x = 166 g.ai ha^{-1}). For each seed population, 240 seedlings were exposed to each

herbicide*dose combination. Three standard susceptible populations were included – BR being susceptible to both ACCase and ALS modes of action, ALOMY_FRA07019 being ALS susceptible and ALOMY_GBH05001 being ACCase susceptible. Thirty two seeds were sown into each of 15 FP11 (L x W x H = 11x11x13cm) pots filled with a 2:1:1 mix of J. Arthur Bower's topsoil (English loam blended with organic matter and nutrients, pH: 6.5 – 7.5), 0.5 Levington growing media: M2 (pH: 5.5 – 6, N: 200, P: 150, K: 200 mg/liter), and 0.25 J. Arthur Bower's silver sand (lime-free washed silica sand). Individual pots were then placed into FP11 potholders, such that each potholder contained one pot for each population collected in a single survey year (Figure 3.2).

Figure 3.2: 15 pots, one for each population sampled in 2012, randomised within a potholder

Populations were randomly allocated positions in the potholder. Each potholder was randomly assigned a herbicide/dose combination and there were 15 potholders for each combination (total number of pot holders was 90, being 15 replicates*6 herbicide/dose combinations). Potholders were arranged in five blocks in a split plot design with main plots being blocks and sub plots being herbicide/dose combinations. Each block contained 3 herbicide/dose sub plots (potholders) and all

potholders were randomly arranged within a block. Pots were maintained in the glasshouse (conditions: 22°C day/16°C night; 14 hour photoperiod) and regularly watered until herbicide application; fertiliser was applied when required. Three weeks after sowing, plants were thinned to 16 plants per pot before being treated with the required dose of mesosulfuron-methyl + iodosulfuron-methyl-sodium or fenoxaprop-p-ethyl. Herbicide was applied using a Berthoud 2000 knapsack sprayer. The sprayer was set to apply herbicide at a pressure of 300 kPa while walking at a fixed pace of 3 kph. A spray volume of 200L ha^{-1} was delivered through a Hypro standard flat fan tip (110 degrees F110-03 ultra-blue nozzle), positioned 40cm above the height of the modular tray. Plants were returned to the glasshouse compartment and watered regularly after herbicide application. Twenty-one days after herbicide application the number of plants surviving herbicide treatment was recorded. This protocol was followed for all three sampling years.

3.3.4 Estimating the frequency of target-site resistance and enhanced metabolism

3.3.4.1 Plant cloning

To determine the presence and frequency of target-site and/or enhanced metabolism mechanisms for ALS and ACCase resistance in annually resampled populations, approximately 25 seeds per sample were sown into a 8x8cm jiffy pot containing a soil mixture of loamy silt soil (LSI: 19% sand, 60% silt, 22% clay, 2.2% organic matter: pH 7.4), Once the seeds were sown, they were covered with approximately 0.5mm of Quartz sand RQ 16 (0.9-2.0 mm). Pots were arranged in polypropylene trays (L x W x H = 50x25x5cm), ten per tray and placed in a climate controlled glasshouse compartment. The glasshouse (temperature: 22.0°C/16°C; photoperiod: 14 hours; humidity: 50%) was equipped with supplementary lighting (turned on

when natural light intensity < 15 kilolux (bulbs: Philips Son-T Agro (400 W)) and an automatic shading system (activated when the light intensity exceeds 55 kilolux). All pots were watered via the trays as required. Once sixteen plants per sample had reached the three-tiller stage, plants were separated into individual tillers (clones). The cloning process involved removing the plant from the soil, separating the tillers at the shoots and roots using a scalpel, and cutting shoots to approximately 3cm in length. Cloned tillers were re-planted into fresh jiffy pots filled with the soil mixture described above and stood in fresh polypropylene trays. This process was repeated until each of the sixteen original plants had a minimum of five clones. Each of the five clones was used to identify the presence of ALS and ACCase target-site and enhanced metabolic resistance as described below.

3.3.4.2 Analysis of Target-site mutations and enhanced metabolism

For each of the sixteen plants per population sample, a 2cm section of leaf tissue was excised from the clone used as the untreated phenotyping control (see section 3.3.5.1) and the presence and frequency of target site mutations (two ALS and five ACCase) was assessed using the PCR and pyrosequencing method described in section 2.3.3. To assess the level of mesosulfuron (ALS) and fenoxaprop (ACCase) enhanced metabolism in each of the sixteen plants per annual population sample, two clones, one for mesosulfuron and one for fenoxaprop metabolism, were assessed using high-performance liquid chromatography using the method described in section 2.3.4.

3.3.4.3 Confirmation of resistance mechanisms by phenotyping

To ensure that the mechanisms of resistance identified through PCR and

pyrosequencing endowed resistance, three of the five clones produced were reserved for phenotyping. One clone was treated with the UK recommended field dose of mesosulfuron-methyl + iodosulfuron-methyl-sodium (12 + 2.4 g.ai ha^{-1}) and the adjuvant Biopower (0.27 kg a.i ha^{-1}), and one clone with the UK recommended field dose of fenoxaprop-p-ethyl (83 g.ai ha^{-1}). The third clone was used as an untreated control for comparison. There were 16 plants phenotyped for each population in each sample year. The 16 plants were arranged in four blocks within a glasshouse compartment, with 4 plants per population randomly assigned to each block. Plant*treatment combinations were randomly arranged in a fully randomised block design. Herbicide was applied at a volume of 300 l ha^{-1} and a pressure of 200 kPa using a track sprayer fitted with a Teejet XR8002 nozzle. The nozzle was positioned approximately 35 – 40 cm from the median plant height. After herbicide application, 5-6 ml (equivalent to approximately 100 kg N ha^{-1}) of the liquid fertilizer (Wuxal Super: N8 – P8 – K6, citrus fertilizer) was applied. Twenty-one days after herbicide application, the mortality of every individual was determined it's to establish resistance phenotype.

3.3.5 Data analysis and presentation

3.3.5.1 Phenotype data

The proportion of individuals resistant to each of the three doses of the ALS herbicide mesosulfuron-methyl + iodosulfuron-methyl-sodium, and the ACCase herbicide fenoxaprop-p-ethyl were translated into a single resistance index for each mode of action. This index was calculated for each MOA by weighting the proportion survival at each dose by the dose applied, so that survival at higher doses contributes more to the resistance index (RI). The scores for each MOA was then

normalised by dividing by 3.5, so that the highest attainable score if all plants survive at all doses is one (i.e. RI = 1) (Equation 3.1).

$$\frac{((\text{survival at } 0.5x*0.5)+(\text{survival at } 1x*1)+(\text{survival at } 2x*2))}{3.5}$$

(Equation 3.1)

3.3.5.2 Target-site resistance and enhanced metabolism data

The allele frequency for each ALS (Pro-197, Trp-574) and ACCase (Ile-1781, Trp-2027, Ile-2041, Asp-2078, Gly-2096) mutation based on target-site genotyping of 16 individuals for each population sample/year was calculated. HPLC analysis of herbicide metabolites produced data exhibiting the proportion of herbicide metabolized on a per plant basis. Based on the proportion of herbicide metabolized, each of 16 individuals were classified as exhibiting low (could metabolize 0 – 20% of the herbicide), intermediate (could metabolize 21 – 49% of the herbicide) and high (could metabolize 50 – 100% of the herbicide) metabolism for ALS and ACCase herbicide mesosulfuron and fenoxaprop respectively. The proportion of plants in each category for each population*year was calculated.

3.3.5.3 Management data

The field management history for the eight-year period (2007 – 2014) was represented as: Crop sown, sowing season (winter or spring), sowing date (early autumn = before October 1st, mid-autumn = between October 1st and October 20th, late autumn = after 20th October, early spring = before April 15th, late spring = after April 15th), cultivation (ploughed or minimum tillage), glyphosate application (glyphosate or none), pre-emergence herbicide applied ("a + b" = active ingredients applied as separate herbicides, "(a + b)" = active ingredients applied as a single

mixed formulation) and post-emergence herbicide applied ("a + b" = active ingredients applied as separate herbicides, "(a + b)" = active ingredients applied as a mixed formulation).

3.3.5.4 Statistical analysis of resistance and mechanism change

To assess how ALS and ACCase phenotypic resistance changes across all seventeen populations between the three years, the phenotype index (as calculated in section 3.3.4.1) was averaged for all populations for each of the years was and compared using a Fisher's exact test to identify any significant changes between them. The average ALS and ACCase allele frequencies, and average proportion of ALS (mesosulfuron) and ACCase (fenoxaprop) metabolites for all population's mutations in each of the three years were also compared using a Fisher's exact test.

3.4 Results

3.4.1 Population data summaries

The phenotype, target-site, enhanced metabolism and management data was compiled and presented in a series of tables, for each of the seventeen populations sampled between 2011 and 2014 (Tables 3.1 – 3.17). As mentioned previously (section 3.2), the results presented here are descriptions of the populations studied. Subsequent epidemiological analysis (chapter 4) will determine how the frequency of ALS and ACCase resistance changes in relation to weed management.

Table 3.1: ALOMY_GBR_xx066. As part the survey conducted in 2011, the phenotype of the population was determined as either resistant (R) or susceptible (S). The resistance index for ALS or ACCase MOA in 2012, 2013, and 2014 is represented in red on the pie chart. In the ALS TSR pie chart, the proportion of Pro-197 (blue), Trp-574 (yellow), both mutations together (blue and yellow stripe), and wildtype (green) target-site mutations are indicated. Similarly, for the ACCase TSR pie chart, the proportion of Ile-1781 (blue), Trp-2027 (orange), Ile-2041 (yellow), Asp-2078 (red), Gly-2096 (black), and wildtype (green) target-site mutations are presented - striped combinations represent these mutations occurring together in the same individual. ALS and ACCase enhanced metabolism, represented by the EMR pie charts, indicate the proportion of individuals with low (green - could metabolise 0 – 20% of the herbicide), intermediate (orange - could metabolise 21 – 49% of the herbicide), and high (red - could metabolise 50 – 100% of the herbicide) metabolic ability. The management history for the eight-year period (2007 – 2014) is represented as: crop sown, season (winter or spring), sowing (early autumn = before October 1st, mid-autumn = between October 1st and October 20th, late autumn = after 20th October, early spring = before April 15th, late spring = after April 15th), cultivation (ploughed or minimum tillage), glyphosate (glyphosate or none), pre-emergence herbicide applied ("a + b" = active ingredients applied as separate herbicides, "(a + b)" = active ingredients applied as part of the same herbicide) and post-emergence herbicide applied ("a + b" = active ingredients applied as separate herbicides, "(a + b)" = active ingredients applied as part of the same herbicide). Any cells within the table left empty (-) indicate no data was collected for that factor in that year. The red shading in each box indicates the level of *A. myosuroides* infesting the field at the time of sampling: white = no black-grass found, a low density of occasional solitary plants, occasional small patches, large patches, widespread throughout the field, a dense and serious infestation.

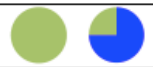
Year	2007		2008		2009		2010		2011		2012		2013		2014	
MOA	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase
Phenotype	-		-		-		-		R	R					-	
TSR	-		-		-		-								-	
EMR	-		-		-		-								-	
Crop	Wheat		Wheat		Oilseed Rape		Wheat		Beans		Wheat		Wheat		Linseed	
Season	Winter		Winter		Winter		Winter		Winter		Winter		Winter		Spring	
Sowing	Early		Mid		Early		Early		Late		Mid		Late		Late	
Cultivation	Min til		Plough		Min til		Min til		Plough		Min til		Plough		Min til	
Glyphosate	None		None		None		None		Glyphosate		Glyphosate		Glyphosate		Glyphosate	
Pre-em	Isoproturon (C2) Trifluralin (K1)		Isoproturon (C2) Pendimethalin (K1)		None		(Flufenacet (K3) + Pendimethalin (K1))		None		(Flufenacet (K3) + Pendimethalin (K1))		None		None	
Post-em	(Mesosulfuron (B) + Iodosulfuron (B))		(Mesosulfuron (B) + Iodosulfuron (B))		Tepraloxydim (A) Cycloxydim (A)		(Mesosulfuron (B) + Iodosulfuron (B))		Tepraloxydim (A)		(Mesosulfuron (B) + Iodosulfuron (B))		(Mesosulfuron (B) + Iodosulfuron (B))		Tepraloxydim (A)	

Table 3.2: ALOMY_GBR_xx069. Refer to Table 3.1 for details.

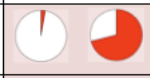
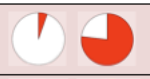
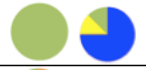
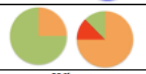
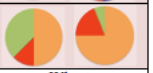
Year	2007		2008		2009		2010		2011		2012		2013		2014	
MOA	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**
Phenotype	-		-		-		-		S	R						
TSR	-		-		-		-									
EMR	-		-		-		-									
Crop	Beans		Wheat		Sugar beet		Wheat		Wheat		Wheat		Sugar beet		Wheat	
Season	Winter		Winter		Spring		Winter		Winter		Winter		Spring		Winter	
Sowing	Late		Early		Early		Late		Mid		Mid		Early		Mid	
Cultivation	Plough		Min til		Plough		Min til		Plough		Min til		Plough		Min til	
Glyphosate	Glyphosate		None		None		None		None		None		None		None	
Pre-em	Simazine (C1) Trifluralin (K1)		Pendimethalin (K1)		None		Diflufenican (K3) Pendimethalin (K1)		None		(Flufenacet (K3) + Pendimethalin (K1)) Diflufenican (K3)		None		(Flufenacet (K3) + Pendimethalin (K1)) Diflufenican (K3)	
Post-em	None		None		None		None		None		None		None		None	

Table 3.2: ALOMY_GBR_xx070. Refer to Table 3.1 for details.

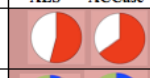
Year	2007		2008		2009		2010		2011		2012		2013		2014	
MOA	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**
Phenotype	-		-		-		-		R	R						
TSR	-		-		-		-									
EMR	-		-		-		-									
Crop	Wheat		Beans		Wheat		Sugar beet		Wheat		Wheat		Sugar beet		Wheat	
Season	Winter		Winter		Winter		Spring		Winter		Winter		Spring		Winter	
Sowing	Late		Late		Mid		Early		Mid		Early		Early		Mid	
Cultivation	Min til		Plough		Min til		Plough		Min til		Plough		Plough		Min til	
Glyphosate	None		None		None		None		None		None		None		None	
Pre-em	None		Trifluralin (K1) Pendimethalin (K1)		Diflufenican (K3) Isoproturon (C2)		None		(Flufenacet (K3) + Pendimethalin (K1)) Diflufenican (K3)		(Flufenacet (K3) + Pendimethalin (K1)) Diflufenican (K3)		None		(Flufenacet (K3) + Pendimethalin (K1)) Diflufenican (K3)	
Post-em	(Mesosulfuron (B) + Iodosulfuron (B))		Tepraloxydim (A)		(Mesosulfuron (B) + Iodosulfuron (B))		None		(Mesosulfuron (B) + Iodosulfuron (B))		(Mesosulfuron (B) + Iodosulfuron (B))		None		(Mesosulfuron (B) + Iodosulfuron (B))	

Table 3.4: ALOMY_GBR_xx075. Refer to Table 3.1 for details.

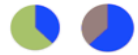
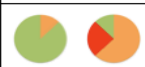
Year	2007		2008		2009		2010		2011		2012		2013		2014	
MOA	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**
Phenotype	-		-		-		-		R	R						
TSR	-		-		-		-									
EMR	-		-		-		-									
Crop	Oilseed rape		Wheat		Wheat		Oilseed rape		Wheat		Wheat		Oilseed rape		Wheat	
Season	Winter		Winter		Winter		Winter		Winter		Winter		Winter		Winter	
Sowing	Early		Early		Mid		Early		Mid		Early		Early		Early	
Cultivation	Min til		Min til		Min til		Min til		Min til		Min til		Min til		Min til	
Glyphosate	Glyphosate		Glyphosate		Glyphosate		Glyphosate		Glyphosate		Glyphosate		Glyphosate		Glyphosate	
Pre-em	Propyzamide (K1)		(Flufenacet (K3) + Diflufenican (K3)) (Flufenacet (K3) + Pendimethalin (K1)) Tri-allate (N)		(Flufenacet (K3) + Diflufenican (K3)) (Flufenacet (K3) + Pendimethalin (K1)) Tri-allate (N)		Propyzamide (K1)		(Flufenacet (K3) + Diflufenican (K3)) (Flufenacet (K3) + Pendimethalin (K1)) Tri-allate (N)		(Flufenacet (K3) + Diflufenican (K3)) (Flufenacet (K3) + Pendimethalin (K1)) Tri-allate (N)		Propyzamide (K1)		(Flufenacet (K3) + Diflufenican (K3)) (Flufenacet (K3) + Pendimethalin (K1)) Tri-allate (N)	
Post-em	Propaquizafop (A)		(Mesosulfuron (B) + Iodosulfuron (B))		(Mesosulfuron (B) + Iodosulfuron (B))		Propaquizafop (A)		(Mesosulfuron (B) + Iodosulfuron (B))		(Mesosulfuron (B) + Iodosulfuron (B))		Propaquizafop (A)		(Mesosulfuron (B) + Iodosulfuron (B))	

Table 3.5: ALOMY_GBR_xx091. Refer to Table 3.1 for details.

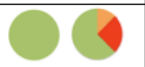
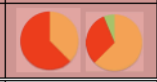
Year	2007		2008		2009		2010		2011		2012		2013		2014	
MOA	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**
Phenotype	-		-		-		-		S	R					-	
TSR	-		-		-		-								-	
EMR	-		-		-		-								-	
Crop	Wheat		Oilseed rape		Wheat		Wheat		Beans		Wheat		Wheat		Oilseed rape	
Season	Winter		Winter		Winter		Winter		Spring		Winter		Winter		Winter	
Sowing	Early		Early		Early		Early		Early		Early		Mid		Early	
Cultivation	None		None		Min til		Min til		Plough		Min til		None		None	
Glyphosate	Glyphosate		None		Glyphosate		None		Glyphosate		None		Glyphosate		None	
Pre-em	(Flufenacet (K3) + Diflufenican (K3)) Trifluralin (K1)		None		None		(Flufenacet (K3) + Diflufenican (K3))		(Pendimethalin (K1) + Imazamox (B))		Diflufenican (K3)		None		Metzachlor (K3) Propyzamide (K1)	
Post-em	(Mesosulfuron (B) + Iodosulfuron (B))		Propaquizafop (A) Tepraloxydim (A)		(Mesosulfuron (B) + Iodosulfuron (B))		(Mesosulfuron (B) + Iodosulfuron (B))		None		(Mesosulfuron (B) + Iodosulfuron (B))		(Mesosulfuron (B) + Iodosulfuron (B))		Clethodim (A)	

Table 3.6 ALOMY_GBR_xx092. Refer to Table 3.1 for details.

Year	2007		2008		2009		2010		2011		2012		2013		2014	
MOA	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase
Phenotype	-		-		-		-		R	R			-			
TSR	-		-		-		-						-			
EMR	-		-		-		-						-			
Crop	Oilseed rape		Wheat		Wheat		Oilseed rape		Wheat		Wheat		Beans		Wheat	
Season	Winter		Winter		Winter		Winter		Winter		Winter		Spring		Winter	
Sowing	Early		Early		Mid		Early		Early		Early		Early		Early	
Cultivation	Min til		Min til		Min til		Min til		Min til		Min til		None		None	
Glyphosate	None		Glyphosate		None		Glyphosate		Glyphosate		Glyphosate		Glyphosate		None	
Pre-em	None		Trifluralin (K1)		None		Propyzamide (K1) Metzachlor (K3)		None		(Flufenacet (K3) + Diflufenican (K3)) Pendimethalin (K1)		(Metzachlor (K3) + Imazamox (B))		(Flufenacet (K3) + Diflufenican (K3)) (Diflufenican (K3) + Flupyrsulfuron (B))	
Post-em	Propaquizafop (A)		(Mesosulfuron (B) + Iodosulfuron (B))		(Mesosulfuron (B) + Iodosulfuron (B))		Propaquizafop (A)		(Mesosulfuron (B) + Iodosulfuron (B))		(Mesosulfuron (B) + Iodosulfuron (B))		Tepraloxydim (A)		(Mesosulfuron (B) + Iodosulfuron (B))	

Table 3.7 ALOMY_GBR_xx096. Refer to Table 3.1 for details.

Year	2007		2008		2009		2010		2011		2012		2013		2014	
MOA	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase
Phenotype	-		-		-		-		R	R					-	
TSR	-		-		-		-								-	
EMR	-		-		-		-								-	
Crop	-		-		-		-		-		-		-		-	
Season	-		-		-		-		-		-		-		-	
Sowing	-		-		-		-		-		-		-		-	
Cultivation	-		-		-		-		-		-		-		-	
Glyphosate	-		-		-		-		-		-		-		-	
Pre-em	-		-		-		-		-		-		-		-	
Post-em	-		-		-		-		-		-		-		-	

Table 3.8 ALOMY_GBR_xx105 Refer to Table 3.1 for details.

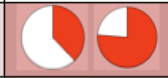
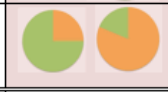
Year	2007		2008		2009		2010		2011		2012		2013		2014	
MOA	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase
Phenotype	-		-		-		-		R	R						
TSR	-		-		-		-									
EMR	-		-		-		-									
Crop	-		-		-		-		-		-		-		-	
Season	-		-		-		-		-		-		-		-	
Sowing	-		-		-		-		-		-		-		-	
Cultivation	-		-		-		-		-		-		-		-	
Glyphosate	-		-		-		-		-		-		-		-	
Pre-em	-		-		-		-		-		-		-		-	
Post-em	-		-		-		-		-		-		-		-	

Table 3.9 ALOMY_GBR_xx106 Refer to Table 3.1 for details.

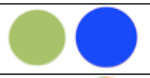
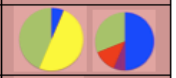
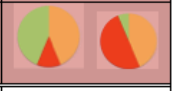
Year	2007		2008		2009		2010		2011		2012		2013		2014	
MOA	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase
Phenotype	-		-		-		-		S	R						
TSR	-		-		-		-									
EMR	-		-		-		-									
Crop	-		-		-		-		-		-		-		-	
Season	-		-		-		-		-		-		-		-	
Sowing	-		-		-		-		-		-		-		-	
Cultivation	-		-		-		-		-		-		-		-	
Glyphosate	-		-		-		-		-		-		-		-	
Pre-em	-		-		-		-		-		-		-		-	
Post-em	-		-		-		-		-		-		-		-	

Table 3.10 ALOMY_GBR_xx110 Refer to Table 3.1 for details.

Year	2007		2008		2009		2010		2011		2012		2013		2014	
MOA	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**
Phenotype	-		-		-		-		S	R						
TSR	-		-		-		-									
EMR	-		-		-		-									
Crop	Oilseed rape		Wheat		Wheat		Peas		Wheat		Barley		Oilseed rape		Wheat	
Season	Winter		Winter		Winter		Spring		Winter		Winter		Winter		Winter	
Sowing	Early		Mid		Early		Early		Early		Early		Early		Early	
Cultivation	Min til		Min til		Min til		Min til		Min til		Min til		Min til		Min til	
Glyphosate	None		None		None		None		None		None		None		None	
Pre-em	Propyzamide (K1)		(Flufenacet (K3) + Diflufenican (K3)) Pendimethalin (K1)		Diflufenican (K3) Pendimethalin (K1)		Pendimethalin (K1)		Flufenacet (K3) Diflufenican (K3)		(Flufenacet (K3) + Pendimethalin (K1)) Flupyrsulfuron (B)		Propyzamide (K1) Metzachlor (K3)		None	
Post-em	None		(Mesosulfuron (B) + Iodosulfuron (B))		(Mesosulfuron (B) + Iodosulfuron (B))		Cycloxydim (A)		(Mesosulfuron (B) + Iodosulfuron (B))		(Mesosulfuron (B) + Iodosulfuron (B))		Propaquizafop (A)		(Mesosulfuron (B) + Iodosulfuron (B))	

Table 3.11 ALOMY_GBR_xx111 Refer to Table 3.1 for details.

Year	2007		2008		2009		2010		2011		2012		2013		2014	
MOA	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**
Phenotype	-		-		-		-		S	R						
TSR	-		-		-		-									
EMR	-		-		-		-									
Crop	Wheat		Wheat		Wheat		Barley		Beans		Wheat		Wheat		Linseed	
Season	Winter		Winter		Winter		Winter		Winter		Winter		Spring		Winter	
Sowing	Early		Early		Mid		Mid		Mid		Mid		Early		Early	
Cultivation	Min til		Min til		Min til		Min til		Plough		Min til		Min til		Min til	
Glyphosate	Glyphosate		None		None		None		None		None		None		None	
Pre-em	(Flufenacet (K3) + Pendimethalin (K1)) (Linuron (C2) + Trifluralin (K1))		Propyzamide (K1)		Trifluralin (K1)		Flupyrsulfuron (B)		Propyzamide (K1) Carbetamide (K2)		(Flufenacet (K3) + Pendimethalin (K1)) Flupyrsulfuron (B)		Diflufenican (K3) Pendimethalin (K1)		(Metzachlor (K3) + Quinmerac (O))	
Post-em	(Mesosulfuron (B) + Iodosulfuron (B)) Pinoxaden (A)		Propaquizafop (A)		(Mesosulfuron (B) + Iodosulfuron (B))		Pinoxaden (A)		None		(Mesosulfuron (B) + Iodosulfuron (B))		(Mesosulfuron (B) + Iodosulfuron (B))		Tepraloxydim (A) Cycloxydim (A)	

Table 3.12 ALOMY_GBR_xx113 Refer to Table 3.1 for details.

Year	2007		2008		2009		2010		2011		2012		2013		2014	
MOA	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase
Phenotype	-		-		-		-		S	R			-			
TSR	-		-		-		-						-			
EMR	-		-		-		-						-			
Crop	-		Wheat		Wheat		Oilseed rape		Wheat		Wheat		Beans		Wheat	
Season	-		Winter		Winter		Winter		Winter		Winter		Spring		Winter	
Sowing	-		Early		Mid		Early		Early		Mid		Early		Mid	
Cultivation	-		None		Min til		None		None		None		Plough		None	
Glyphosate	-		Glyphosate		Glyphosate		None		Glyphosate		Glyphosate		None		Glyphosate	
Pre-em	-		(Flufenacet (K3) + Diflufenican (K3)) Trifluralin (K1)		(Flufenacet (K3) + Diflufenican (K3)) Trifluralin (K1)		(Metzachlor (K3) + Dimethenamid-p (K3) +Quinmerac (O)) Propyzamide (K1)		(Flufenacet (K3) + Diflufenican (K3))		(Flufenacet (K3) + Diflufenican (K3))		(Imazamox (B) + Pendimethalin (K1)) Pendimethalin (K1)		(Flufenacet (K3) + Pendimethalin (K1))	
Post-em	-		(Mesosulfuron (B) + Iodosulfuron (B))		(Mesosulfuron (B) + Iodosulfuron (B))		Propaquizafop (A)		None		(Mesosulfuron (B) + Iodosulfuron (B))		None		(Mesosulfuron (B) + Iodosulfuron (B))	

Table 3.13 ALOMY_GBR_xx115 Refer to Table 3.1 for details.

Year	2007		2008		2009		2010		2011		2012		2013		2014	
MOA	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase
Phenotype	-		-		-		-		R	R	-					
TSR	-		-		-		-				-					
EMR	-		-		-		-				-					
Crop	-		Wheat		Beans		Wheat		Wheat		Oilseed rape		Wheat		Wheat	
Season	-		Winter		Spring		Winter		Winter		Winter		Winter		Winter	
Sowing	-		Early		Early		Early		Mid		Early		Early		Mid	
Cultivation	-		Plough		Plough		None		None		None		None		None	
Glyphosate	-		None		Glyphosate		Glyphosate		Glyphosate		None		Glyphosate		Glyphosate	
Pre-em	-		(Flufenacet (K3) + Diflufenican (K3)) (Flufenacet (K3) + Pendimethalin (K1)) Trifluralin (K1)		Trifluralin (K1) (Imazamox (B) + Pendimethalin (K1))		(Flufenacet (K3) + Diflufenican (K3)) Pendimethalin (K1)		(Flufenacet (K3) + Diflufenican (K3)) Pendimethalin (K1)		(Metzachlor (K3) + Dimethenamid-p (K3) +Quinmerac (O)) Propyzamide (K1)		(Flufenacet (K3) + Diflufenican (K3))		(Flufenacet (K3) + Diflufenican (K3))	
Post-em	-		(Mesosulfuron (B) + Iodosulfuron (B))		None		(Mesosulfuron (B) + Iodosulfuron (B))		None		None		(Mesosulfuron (B) + Iodosulfuron (B))		(Mesosulfuron (B) + Iodosulfuron (B))	

Table 3.14 ALOMY_GBR_xx125 Refer to Table 3.1 for details.

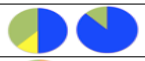
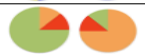
Year	2007		2008		2009		2010		2011		2012		2013		2014	
MOA	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**
Phenotype	-		-		-		-		R	R	-				-	
TSR	-		-		-		-				-				-	
EMR	-		-		-		-				-				-	
Crop	Wheat		Oilseed rape		Wheat		Oilseed rape		Wheat		Oilseed rape		Barley		Oilseed rape	
Season	Winter		Winter		Winter		Winter		Winter		Winter		Spring		Winter	
Sowing	Early		Early		Mid		Early		Early		Early		Early		Early	
Cultivation	None		Min til		Min til		Min til		Min til		Min til		Min til		Min til	
Glyphosate	Glyphosate		None		Glyphosate		None		Glyphosate		None		Glyphosate		None	
Pre-em	(Flufenacet (K3) + Diflufenican (K3)) (Flufenacet (K3) + Pendimethalin (K1)) Trifluralin (K1)		Metzachlor (K3)		(Flufenacet (K3) + Diflufenican (K3)) Trifluralin (K1)		Metzachlor (K3)		(Flufenacet (K3) + Diflufenican (K3)) (Flufenacet (K3) + Pendimethalin (K1)) Prosulfocarb (K1)		Propyzamide (K1) Carbetamide (K2)		None		Propyzamide (K1)	
Post-em	Mesosulfuron (B) Iodosulfuron (B)		Tepraloxydim (A)		Mesosulfuron (B) Iodosulfuron (B)		Propaquizafop (A) Cycloxydim (A)		Mesosulfuron (B) Iodosulfuron (B)		Propaquizafop (A)		None		Propaquizafop (A) Clethodim (A)	

Table 3.15 ALOMY_GBR_xx126 Refer to Table 3.1 for details.

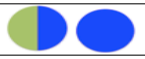
Year	2007		2008		2009		2010		2011		2012		2013		2014	
MOA	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**	**ALS**	**ACCase**
Phenotype	-		-		-		-		R	R						
TSR	-		-		-		-									
EMR	-		-		-		-									
Crop	Oilseed rape		Wheat		Oilseed rape		Wheat		Beans		Wheat		Oilseed rape		Wheat	
Season	Winter		Winter		Winter		Winter		Winter		Winter		Winter		Winter	
Sowing	Early		Early		Early		Early		Mid		Mid		Early		Early	
Cultivation	Min til		None		None		Min til		Min til		Min til		None		Min til	
Glyphosate	None		Glyphosate		None		None		Glyphosate		Glyphosate		Glyphosate		Glyphosate	
Pre-em	Propyzamide (K1)		(Flufenacet (K3) + Diflufenican (K3)) Trifluralin (K1)		None		(Flufenacet (K3) + Diflufenican (K3)) Prosulfocarb (K1)		Prosulfocarb (K1)		(Flufenacet (K3) + Diflufenican (K3)) (Flufenacet (K3) + Pendimethalin (K1)) Prosulfocarb (K1)		None		(Flufenacet (K3) + Diflufenican (K3)) Flupyrsulfuron (B) Tri-allate (N)	
Post-em	Tepraloxydim (A)		(Mesosulfuron (B) + Iodosulfuron (B))		None		(Mesosulfuron (B) + Iodosulfuron (B))		None		(Mesosulfuron (B) + Iodosulfuron (B))		Propaquizafop (A) Clethodim (A)		Pinoxaden (A)	

Table 3.16 ALOMY_GBR_xx133 Refer to Table 3.1 for details.

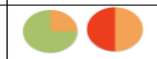
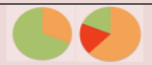
Year	2007		2008		2009		2010		2011		2012		2013		2014	
MOA	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase
Phenotype	-		-		-		-		S	R	-					
TSR	-		-		-		-				-					
EMR	-		-		-		-				-					
Crop	-		-		Oilseed rape		Wheat		Sugar beet		Wheat		Oilseed rape		Wheat	
Season	-		-		Winter		Winter		Spring		Winter		Winter		Winter	
Sowing	-		-		Early		Early		Early		Mid		Early		Mid	
Cultivation	-		-		None		Min til		Plough		Plough		Plough		Min til	
Glyphosate	-		-		Glyphosate		None		Glyphosate		None		Glyphosate		Glyphosate	
Pre-em	-		-		(Metzachlor (K3) + Quinmerac (O)) Propyzamide (K1)		(Flufenacet (K3) + Diflufenican (K3))		None		(Flufenacet (K3) + Diflufenican (K3)) Flupyrsulfuron (B)		(Metzachlor (K3)+ Quinmerac (O)+ Dimethenamid-p (K3)) Propyzamide (K1)		(Flufenacet (K3) + Diflufenican (K3)) Pendimethalin (K1)	
Post-em	-		-		Tepraloxydim (A)		(Mesosulfuron (B) + Iodosulfuron (B))		Tepraloxydim (A)		(Mesosulfuron (B) + Iodosulfuron (B))		Tepraloxydim (A)		(Mesosulfuron (B) + Iodosulfuron (B))	

Table 3.17 ALOMY_GBR_xx135 Refer to Table 3.1 for details.

Year	2007		2008		2009		2010		2011		2012		2013		2014	
MOA	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase	ALS	ACCase
Phenotype	-		-		-		-		S	R						
TSR	-		-		-		-									
EMR	-		-		-		-									
Crop	-		-		-		-		-		-		-		-	
Season	-		-		-		-		-		-		-		-	
Sowing	-		-		-		-		-		-		-		-	
Cultivation	-		-		-		-		-		-		-		-	
Glyphosate	-		-		-		-		-		-		-		-	
Pre-em	-		-		-		-		-		-		-		-	
Post-em	-		-		-		-		-		-		-		-	

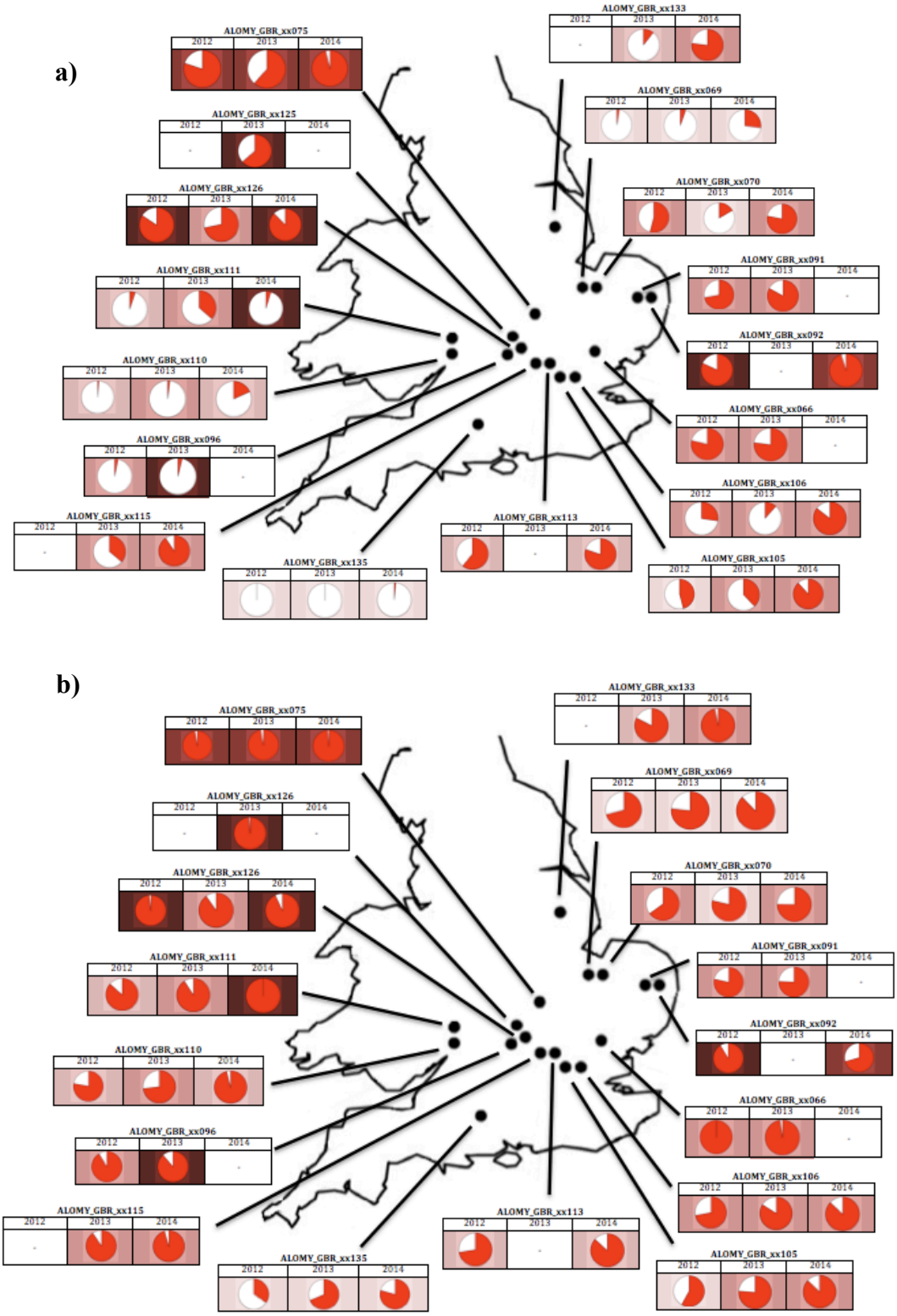


Figure 3.3: ALS (a) and ACCase (b) resistance indexes in the 17 UK populations sampled between 2012 and 2014. The resistance index for ALS or ACCase MOA in 2012, 2013, and 2014 is represented in red on the pie chart. The red shading in each box indicates the level of *A. myosuroides* infesting the field at the time of sampling: white = no black-grass found, a low density of occasional solitary plants, occasional small patches, large patches, widespread throughout the field, a dense and serious infestation.

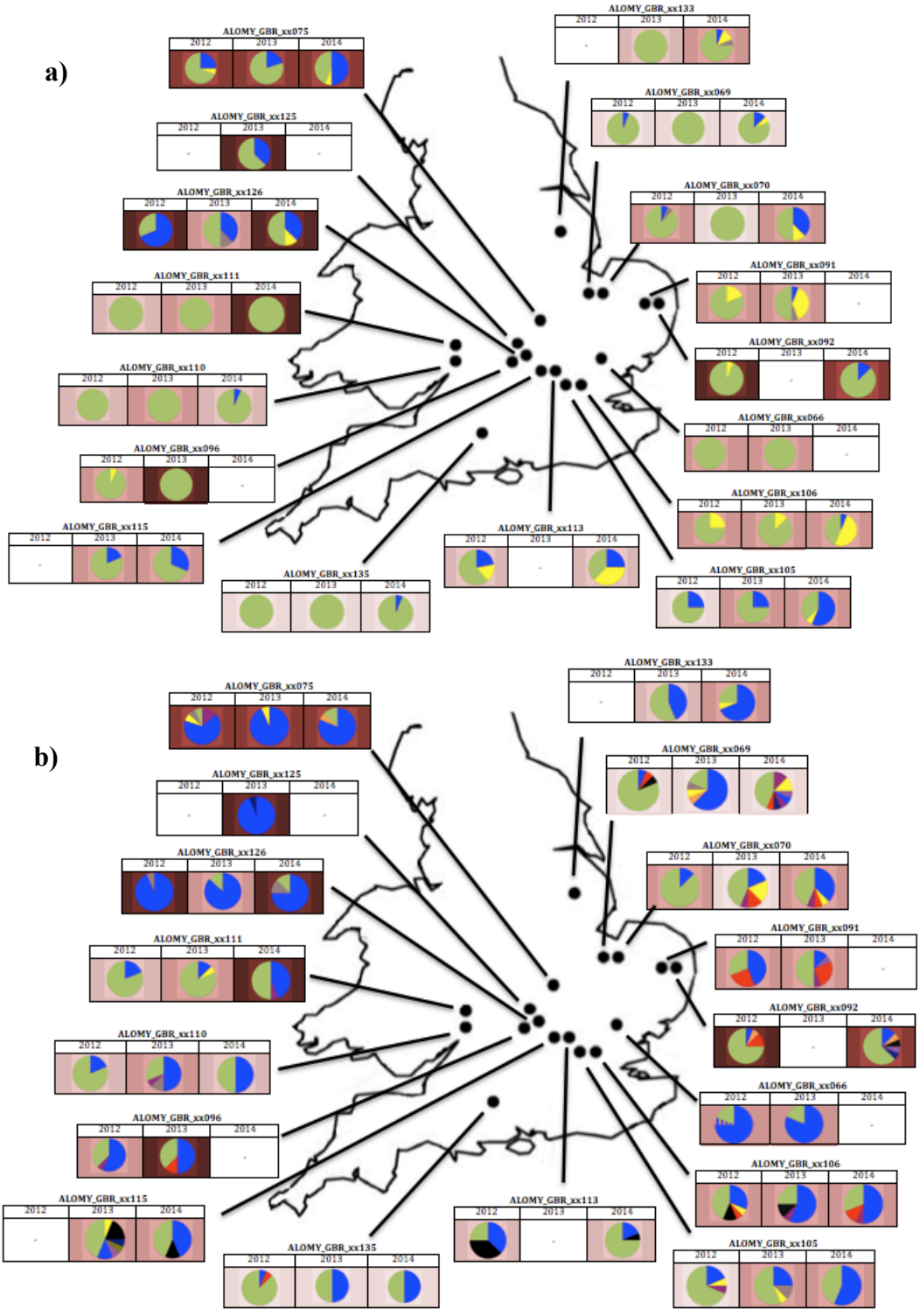


Figure 3.4: Frequencies of ALS (a) and ACCase (b) target-site resistance in the 17 UK populations sampled between 2012 and 2014. In the ALS TSR pie chart, the proportion of Pro-197 (blue), Trp-574 (yellow), both mutations together (blue and yellow stripe), and wildtype (green) target-site mutations are indicated. Similarly, for the ACCase TSR pie chart, the proportion of Ile-1781 (blue), Trp-2027 (orange), Ile-2041 (yellow), Asp-2078 (red), Gly-2096 (black), and wildtype (green) target-site mutations are presented - stripped combinations represent these mutations occurring together in the same individual. The red shading in each box indicates the level of *A. myosuroides* infesting the field at the time of sampling: white = no black-grass found, a low density of occasional solitary plants, occasional small patches, large patches, widespread throughout the field, a dense and serious infestation.

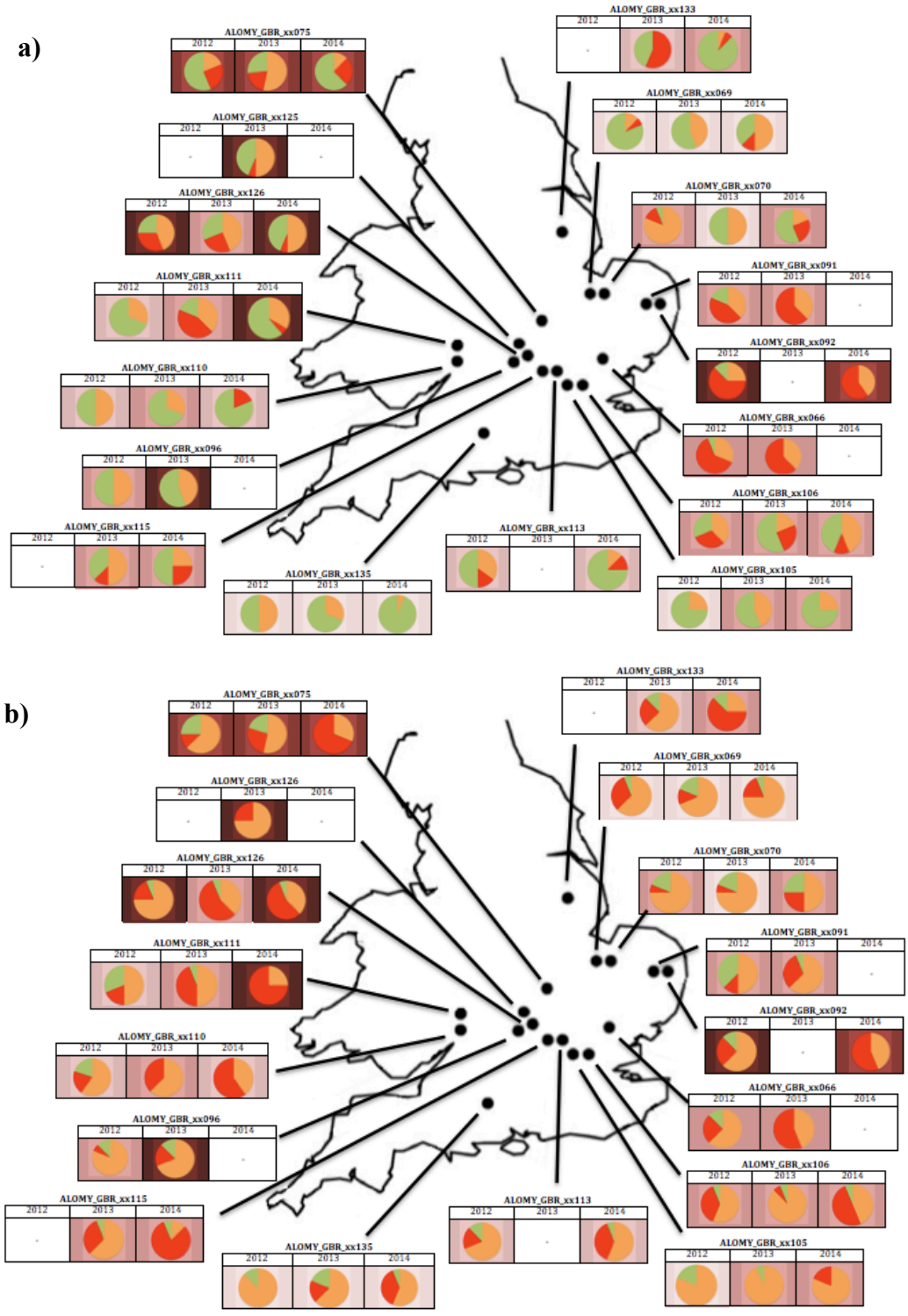


Figure 3.5: Frequencies of ALS (a) and ACCase (b) enhanced metabolism in the 17 UK populations sampled between 2012 and 2014. The proportions of individuals with low (green - could metabolize 0 – 20% of the herbicide), intermediate (orange - could metabolize 21 – 49% of the herbicide), and high (red - could metabolize 50 – 100% of the herbicide) metabolic ability are shown. The red shading in each box indicates the level of *A. myosuroides* infesting the field at the time of sampling: white = no black-grass found, a low density of occasional solitary plants, occasional small patches, large patches, widespread throughout the field, a dense and serious infestation.

3.4.2 Summarizing phenotype, TSR and EMR population trends

The seventeen *A. myosuroides* populations sampled from the UK between 2012 and 2014 exhibited remarkable differences in the frequencies and changes in phenotypic resistance (Figure 3.3), target-site resistance (Figure 3.4) and enhanced metabolism (Figure 3.5) to ALS and ACCase modes of action as described below.

ALOMY_GBR_ xx066: The frequency of ALS phenotypic resistance remained high between 2012 (79%) and 2013 (77%), with no ALS TSR identified in the population in either year of sampling. The number of individuals exhibiting intermediate or high ALS EMR increased between 2012 (94%) and 2013 (100%). The frequency of phenotypic ACCase resistance remained high between 2012 (100%) and 2013 (98%), while the frequency ACCase TSR increased from 56% in 2012 to 72% in 2013. The number of individuals exhibiting intermediate or high ACCase EMR increased between 2012 (87%) and 2013 (100%).

ALOMY_GBR_ xx069: ALS phenotypic resistance steadily increased from 3% in 2012 to 27% in 2014. ALS TSR frequency decreased between 2012 (3%) and 2013 (0%) before increasing again in 2014 (9%), while ALS EMR steadily increased between 2012 (high + intermediate = 19%) and 2014 (high + intermediate = 63%). ACCase phenotypic resistance increased between 2012 (71%) and 2014 (88%), as did the frequency of ALS TSR from 9% in 2012 to 69% in 2014. ALS EMR remained consistently frequent throughout, with high and intermediate EMR individuals equaling 94%, 87% and 94% of the population in 2012, 2013 and 2014 respectively.

ALOMY_GBR_ xx070: ALS resistance decreased between 2012 (54%) and 2013 (17%) before increasing again in 2014 (78%), as did ALS TSR (2012 = 9%, 2013 = 0%, 2014 = 31%). ALS enhanced metabolism decreased, with high + intermediate EMR individuals equaling 94%, 50% and 44% of the population in 2012, 2013 and 2014 respectively. ACCase phenotypic resistance increased between 2012 (65%) and 2013 (79%) before decreasing in 2014 (75%). ACCase TSR increased between 2012 (6%) and 2014 (47%), while ACCase EMR remained constant in 2012 and 2013 (high + intermediate EMR = 81%) before decreasing (high + intermediate EMR = 50%).

ALOMY_GBR_ xx075: ALS resistance decreased between 2012 (80%) and 2013 (61%) before increasing in 2014 (96%), as did ALS TSR (2012 = 20%, 2013 = 10%, 2014 = 31%). ALS EMR increased between 2012 (high + intermediate EMR = 44%) and 2013 (high + intermediate EMR = 81%) before decreasing in 2014 (high + intermediate EMR = 31%) The frequency of ACCase phenotypic resistance remained high (2012 (97%), 2013 (97%), 2014 (99%)), as did the frequency of ACCase TSR (2012 (73%), 2013 (90%), 2014 (73%)). ACCase EMR increased from 75% (high + intermediate EMR) in 2012 to 100% (high + intermediate EMR) in 2014.

ALOMY_GBR_ xx091: ALS Phenotypic resistance increased between 2012 (72%) and 2013 (83%), as did the frequency of ALS TSR (2012 = 9%, 2013 = 28%) and the number of individuals exhibiting high or intermediate ALS EMR (2012 = 81%, 2013 = 100%). ACCase phenotypic resistance remained high between 2012 (78%) and 2013 (76%), as did the frequency of ACCase TSR (2012 = 34%, 2013 = 31%).

However, the number of individuals exhibiting high or intermediate ACCase EMR increased between 2012 (62%) and 2013 (94%).

ALOMY_GBR_ xx092: ALS resistance increased between 2012 (81%) and 2014 (95%). ALS TSR also increased slightly between 2012 (3%) and 2014 (6%), with individuals exhibiting high or intermediate ALS EMR remaining at a high frequency (2012 = 87%, 2014 = 100%). ACCase resistance decreased slightly between 2012 (91%) and 2014 (71%), although ACCase TSR increased (2012 = 13%, 2014 = 34%), as did the number of individuals exhibiting high or intermediate ACCase EMR (2012 = 87%, 2014 = 100%).

ALOMY_GBR_ xx096: The frequency of ALS resistance remained low in 2012 (3%) and 2014 (4%). the frequency of ALS TSR also remained low (3% and 0% in 2012 and 2013 respectively), as did EMR, with 50% and 44% of individuals exhibiting intermediate EMR in 2012 and 2013 respectively. ACCase resistance remained high throughout (2012 = 91%, 2013 = 89%). ACCase TSR remained at 43%, while 81% and 87% of individuals in 2012 and 2013 respectively possessed wither high or intermediate ACCase EMR.

ALOMY_GBR_ xx105: ALS resistance started at 46% in 2012, decreasing to 38% in 2013 before increasing to 88% in 2014. ALS TSR increased from 12% in 2012 to 43% in 2014. The frequency of high or intermediate ALS EMR increased between 2012 (25%) and 2013 (44%) before increasing in 2014 (25%). ACCase resistance and TSR increased between 2012 and 2014 (57 - 87% and 21 – 44% respectively),

while the frequency of individuals with high or intermediate ACCase EMR remained above 94% in all three years.

ALOMY_GBR_ xx106: ALS resistance decreased between 2012 (27%) and 2013 (11%) before increasing again in 2014 (84%). ALS TSR increased between 2012 (6%) and 2014 41%), while the frequency of individuals with high and intermediate ALS EMR decreased between 2012 (68%) and 2013 (44%) before increasing in 2014 (56%). ACCase resistance was high and increased between 2012 and 2014 (72 - 87%). The frequency of ACCase TSR increased over the three-year period (31-53%), while the frequency of individuals with high and intermediate ACCase EMR remained at 94%.

ALOMY_GBR_ xx110: ALS resistance remained low in 2012 and 2013 (<2%), before increasing to 18% in 2014. The frequency of ALS TSR alleles was 0% in 2012 and 2013, before increasing to 3% in 2014. The frequency of individuals with high and intermediate ALS EMR decreased from 50% in 2012 to 19% in 2014. ACCase resistance increased from 77% in 2012 to 96% in 2014. The frequency of ACCase TSR alleles was approximately 10% in 2012, increasing to 50% 2013 before decreasing to 34% in 2014. The frequency of individuals with high and intermediate ACCase EMR increased from 75% in 2012 to 100% in 2013 and 2014.

ALOMY_GBR_ xx111: ALS resistance increased between 2012 (5%) and 2013 (36%) before decreasing in 2014 (4%). The frequency of ALS TSR alleles was 0% in 2012 and 2013 before increasing to 3% in 2014. The frequency of individuals with high and intermediate ALS EMR increased from 31% in 2012 to 81% in 2013 before

decreasing to 44% in 2014. ACCase resistance steadily increased between 2012 (87%) and 2014 (100%), as did the frequency ACCase TSR alleles (9 - 40%) and ACCase EMR (81% - 100%).

ALOMY_GBR_ xx113: ALS phenotypic resistance increased from 60% in 2012 to 80% in 2014; the frequency of ALS TSR alleles increased from 22% to 34% over the same period. The frequency of individuals with high and intermediate ALS EMR remained decreased from 62% in 2013 to 25% in 2014. ACCase resistance increased from 72% in 2012 to 87% in 2014, while the frequency ACCase TSR alleles decreased from 37% to 12%. The frequency of individuals with high and intermediate ACCase EMR increased from 87% in 2012 to 94% in 2014.

ALOMY_GBR_ xx115: ALS resistance increased from 36% in 2013 to 91% in 2014; the frequency of ALS TSR alleles also increased from 1% in 2013 to 20% in 2014. The frequency of individuals with high and intermediate ALS EMR decreased from 64% in 2013 to 50% in 2014. ACCase resistance remained above 90%, while the frequency ACCase TSR alleles remained at 34%. The frequency of individuals with high and intermediate ACCase EMR remained at 94% in 2013 and 2014.

ALOMY_GBR_ xx125: This population was only sampled in one year (2013). The frequency of ALS resistance was 63%. ALS TSR was 20%, and high or intermediate ALS EMR was 56%. The frequency of ACCase resistance was 31%. ACCase TSR was 98%, and high or intermediate ACCase EMR was 100%.

ALOMY_GBR_ xx126: ALS resistance decreased between 2012 (84%) and 2013 (71%) before increasing in 2014 (87%), while ALS TSR alleles remained at frequency of 34% in all three years. The frequency of individuals with high and intermediate ALS EMR remained at 75% in 2012 and 2013, before decreasing to 56% in 2014. ACCase resistance remained above 90% in all years. The frequency ACCase TSR alleles declined from 90% in 2012 to 75% in 2014, while the frequency of individuals with high and intermediate ACCase EMR remained at 94% in all years.

ALOMY_GBR_ xx133: ALS resistance increased between 2013 (10%) and 2014 (77%). ALS TSR alleles were absent in 2013, while the number of individuals exhibiting high and intermediate ALS EMR increased from 13% in 2013 to 56% in 2014. ACCase resistance increased between 2013 (82%) and 2014 (96%). ACCase target-site alleles increased from 28% in 2013 to 50% in 2014, while the number of individuals exhibiting high and intermediate ACCase EMR remained constant at 87%.

ALOMY_GBR_ xx135: ALS resistance remained at 0% in 2012 and 2013; in 2014, phenotypic resistance increased 1%. No target-site resistance alleles were detected in 2012 and 2013, with allele frequency rising to 6% in 2014. The frequency of intermediate ALS EMR decreased between 2012 (50%) and 2014 (6%). The frequency of ACCase resistance increased from 35% in 2012 to 79% in 2014. The frequency of ACCase TSR was 6% in 2012, increasing to 31% in 2014, while the frequency of high and intermediate ACCase EMR decreased from 87% in 2012 to 81% in 2013, before increasing again in 2014 to 94%

3.4.3 Average change in resistance (2012 – 2014)

3.4.3.1 Average change ALS phenotype, TSR and EMR

In 2014, the proportion of mesosulfuron-methyl + iodosulfuron-methyl-sodium resistant individuals across all sampled populations was significantly (Fisher's exact test, $P < 0.05$) higher than that identified in 2012 and 2013; the decrease in mesosulfuron-methyl + iodosulfuron-methyl-sodium resistance between 2012 and 2013 was non-significant (Table 3.18).

Table 3.18: Average ALS phenotypic resistance index, ALS TSR allele frequency and ALS (mesosulfuron) metabolized all populations sampled in each year. The average ALS phenotypic resistance index, ALS TSR allele frequency and ALS EMR from all sampled populations are indicated. The year 2014 is marked with an asterisk (*) for resistance index and TSR allele frequency to indicate a significant (Fisher's exact test, $P < 0.05$) increase between it and the previous two years.

Year	2012	2013	2014
Resistance index	0.42	0.34	0.64*
TSR allele frequency	0.09	0.10	0.21*
Mesosulfuron metabolized	0.33	0.30	0.23

The frequency of ALS target-site resistance alleles remained at a similar level between 2012 and 2013. The total frequency of ALS target-site resistance alleles in 2014 significantly (Fisher's exact test, $P < 0.05$) increases to when compared to the two previous years (Table 3.18). The average amount of ALS herbicide (mesosulfuron) metabolized across all populations exhibited no significant change in the average level across all populations between 2012, 2013 and 2014 (Table 3.18); however, the average level of ALS enhanced metabolism did decrease over the three-year period studied (Table 3.18). Even though the general trends in resistance index, TSR allele frequency, and mesosulfuron metabolised were observed across all populations between 2012 and 2014 (Table 3.18), each population exhibited their own unique response in resistance index, TSR allele frequency, and amount of mesosulfuron metabolised (Table 3.19).

Table 3.19: Changes in ALS resistance index, ALS TSR allele proportion, and mesosulfuron (ALS) metabolised for each of the seventeen UK *A. myosuroides* populations sampled in 2012, 2013 and 2014. Missing data (-) was not included in subsequent analysis and modelling.

Population	Resistance measure	Years 2012	2013	2014
xx066	Resistance index	0.79	0.77	-
	TSR allele prop^n	0.00	0.00	-
	ALS metabolised	0.59	0.62	-
xx069	Resistance index	0.03	0.05	0.27
	TSR allele prop^n	0.03	0.00	0.09
	ALS metabolised	0.21	0.19	0.28
xx070	Resistance index	0.54	0.17	0.78
	TSR allele prop^n	0.09	0.00	0.31
	ALS metabolised	0.32	0.20	0.23
xx075	Resistance index	0.80	0.61	0.96
	TSR allele prop^n	0.20	0.10	0.31
	ALS metabolised	0.30	0.36	0.21
xx091	Resistance index	0.72	0.83	-
	TSR allele prop^n	0.09	0.28	-
	ALS metabolised	0.56	0.56	-
xx092	Resistance index	0.82	-	0.95
	TSR allele prop^n	0.03	-	0.06
	ALS metabolised	0.60	-	0.52
xx096	Resistance index	0.03	0.04	-
	TSR allele prop^n	0.03	0.00	-
	ALS metabolised	0.20	0.19	-
xx105	Resistance index	0.46	0.38	0.88
	TSR allele prop^n	0.13	0.13	0.34
	ALS metabolised	0.15	0.19	0.13
xx106	Resistance index	0.27	0.11	0.85
	TSR allele prop^n	0.06	0.35	0.41
	ALS metabolised	0.35	0.25	0.27
xx110	Resistance index	0.01	0.02	0.18
	TSR allele prop^n	0.00	0.00	0.03
	ALS metabolised	0.17	0.16	0.19
xx111	Resistance index	0.05	0.36	0.05
	TSR allele prop^n	0.00	0.00	0.00
	ALS metabolised	0.17	0.47	0.18
xx113	Resistance index	0.60	-	0.80
	TSR allele prop^n	0.22	-	0.34
	ALS metabolised	0.31	-	0.19
xx115	Resistance index	-	0.36	0.91
	TSR allele prop^n	-	0.09	0.20
	ALS metabolised	-	0.27	0.33
xx125	Resistance index	-	0.63	-
	TSR allele prop^n	-	0.22	-
	ALS metabolised	-	0.26	-
xx126	Resistance index	0.84	0.72	0.87
	TSR allele prop^n	0.34	0.32	0.30
	ALS metabolised	0.42	0.39	0.25
xx133	Resistance index	-	0.11	0.77
	TSR allele prop^n	-	0.00	0.22
	ALS metabolised	-	0.24	0.11
xx135	Resistance index	0.00	0.00	0.01
	TSR allele prop^n	0.00	0.00	0.06
	ALS metabolised	0.19	0.16	0.05

Of the 12 populations that had a sample collected in 2012 and 2013, 5, 6 and 1 exhibited an increase, decrease and no change in resistance index respectively (Table 3.19); 2, 5 and 5 populations exhibited an increase, decrease and no change in ALS TSR respectively, and 4, 7 and 1 populations exhibited an increase, decrease and no change in mesosulfuron metabolisation (Table 3.19).

Of the 11 populations that had a sample collected in 2013 and 2014, 10, 1 and 0 exhibited an increase, decrease and no change in resistance index respectively (Table 3.19); 10, 1 and 0 populations exhibited an increase, decrease and no change in ALS TSR respectively, and 5, 6 and 0 populations exhibited an increase, decrease and no change in mesosulfuron metabolisation (Table 3.19).

Of the 11 populations that had a sample collected in 2012 and 2014, 10, 0, and 1 exhibited an increase, decrease and no change in resistance index respectively (Table 3.19); 10, 0 and 1 populations exhibited an increase, decrease and no change in ALS TSR respectively, and 1, 10 and 0 populations exhibited an increase, decrease and no change in mesosulfuron metabolisation (Table 3.19).

Looking more closely at the specific ALS target-site mutations endowing resistance across the populations each years, it was found that Pro-197-Thr alleles were in the greatest frequency in all years, significantly increasing in proportion in 2014 (Table 3.20). All ALS target-site mutations exhibited a significant increase allele frequency in 2014 when compare with 2012 and 2013 (Table 3.20).

Table 3.20: Frequency of ALS target-site alleles. For each year (2012, 2013, 2014), the allele frequency for each mutation has been calculated differences (Fisher's exact test, $P < 0.05$) with a Fisher's exact test are indicated as: ( ) = no significant difference, (*) = 2014 is significantly different from 2012 only, (**) = 2014 is significant different from 2013 only, (***) = 2014 is significantly different from both 2012 and 2013.

Mutation	Resistance allele frequency		
	2012	2013	2014
Pro-197-Thr	0.056	0.052	0.101***
Pro-197-His	0.004	0	0.012**
Pro-197-Leu	0	0	0.004***
Pro-197-Ser	0	0.002	0.014*
Trp-574-Leu	0.033	0.025	0.067***
Total	0.096	0.079	0.180***

3.4.2.2 Average change ACCase phenotype, TSR and EMR

The ACCase resistance index increased between 2012 and 2014, with the index being significantly (Fisher's exact test, $P < 0.05$) higher in 2014 when compared with 2012 and 2013 (Table 3.21).

Table 3.21: Average ACCase phenotypic resistance index, ACCase TSR allele frequency and ACCase (fenoxaprop) metabolized all populations sampled in each year. The average ALS phenotypic resistance index, ACCase TSR allele frequency and ACCase EMR from all sampled populations are indicated. The year 2014 is marked with an asterisk (*) for resistance index and fenoxaprop metabolized to indicate a significant ($P < 0.05$) increase between it and the previous two years.

Year	2012	2013	2014
Resistant index	0.78	0.85	0.96*
TSR allele frequency	0.32	0.46	0.35
Fenoxaprop metabolized	0.37	0.41	0.50*

The average frequency of ACCase target-site resistance alleles across all seventeen populations non-significantly increased between 2012 and 2013, before decreasing again in 2014 (Table 3.21). The average amount of ACCase herbicide (fenoxaprop) metabolised across all populations exhibited a decrease between 2012 and 2014 (Table 3.21), with the average level of fenoxaprop enhanced metabolism being

significantly ($P < 0.05$) lower in 2014 when compared to 2012 and 2013 (Table 3.21).

Of the 12 populations that had a sample collected in 2012 and 2013, 6, 5 and 1 exhibited an increase, decrease and no change in resistance index respectively (Table 3.22); 9, 2 and 1 populations exhibited an increase, decrease and no change in ACCase TSR respectively, and 8, 4 and 0 populations exhibited an increase, decrease and no change in fenoxaprop metabolisation (Table 3.22). Of the 11 populations that had a sample collected in 2013 and 2014, 10, 1 and 0 exhibited an increase, decrease and no change in resistance index respectively (Table 3.22); 10, 1 and 0 populations exhibited an increase, decrease and no change in ACCase TSR respectively, and 11, 0 and 0 populations exhibited an increase, decrease and no change in fenoxaprop metabolisation (Table 3.22). Of the 11 populations that had a sample collected in 2012 and 2014, 9, 2 and 0 exhibited an increase, decrease and no change in resistance index respectively (Table 3.22); 7, 3 and 1 populations exhibited an increase, decrease and no change in ACCase TSR respectively, and 11, 0 and 0 populations exhibited an increase, decrease and no change in fenoxaprop metabolisation (Table 3.22).

Of all of the ACCase target-site mutations endowing resistance across the seventeen populations, Ile-1781-Leu alleles were in the greatest frequency in all years, being significantly increased in proportion in 2013 compared to 2012 and 2014, (Figure 3.23). Similar to Ile-1781-Leu mutations, Ile-2041-Asn and Ile-2041-Val mutations increased in proportion in 2013 compared to 2012 and 2014 (Figure 3.23). Ile-1781-Val, Ile-1781-Thr and Trp-2027-Cys increased between 2012 and 2014, while Asp-2078-Gly and Gly-2096-Ala mutations decreased year-on-year (Figure 3.23).

Table 3.22: Changes in ACCase resistance index, ACCase TSR allele proportion, and mesosulfuron (ACCase) metabolised for each of the seventeen UK *A. myosuroides* populations sampled in 2012, 2013 and 2014. Missing data (-) was not included in subsequent analysis and modelling.

Population	Resistance measure	Years 2012	2013	2014
xx066	Resistance index	1.00	0.98	-
	TSR allele prop^n	0.56	0.72	-
	ACCase metabolised	0.41	0.52	-
xx069	Resistance index	0.71	0.77	0.88
	TSR allele prop^n	0.09	0.59	0.69
	ACCase metabolised	0.38	0.36	0.41
xx070	Resistance index	0.65	0.79	0.75
	TSR allele prop^n	0.06	0.38	0.47
	ACCase metabolised	0.31	0.38	0.41
xx075	Resistance index	0.97	0.97	0.99
	TSR allele prop^n	0.73	0.90	0.73
	ACCase metabolised	0.34	0.39	0.55
xx091	Resistance index	0.78	0.76	-
	TSR allele prop^n	0.34	0.31	-
	ACCase metabolised	0.27	0.41	-
xx092	Resistance index	0.91	-	0.71
	TSR allele prop^n	0.13	-	0.34
	ACCase metabolised	0.37	-	0.52
xx096	Resistance index	0.91	0.89	-
	TSR allele prop^n	0.43	0.44	-
	ACCase metabolised	0.36	0.38	-
xx105	Resistance index	0.58	0.76	0.87
	TSR allele prop^n	0.22	0.30	0.44
	ACCase metabolised	0.36	0.31	0.42
xx106	Resistance index	0.72	0.84	0.87
	TSR allele prop^n	0.31	0.47	0.53
	ACCase metabolised	0.47	0.35	0.50
xx110	Resistance index	0.78	0.73	0.96
	TSR allele prop^n	0.10	0.50	0.34
	ACCase metabolised	0.31	0.48	0.51
xx111	Resistance index	0.87	0.91	1.00
	TSR allele prop^n	0.09	0.09	0.40
	ACCase metabolised	0.44	0.46	0.62
xx113	Resistance index	0.73	-	0.87
	TSR allele prop^n	0.38	-	0.13
	ACCase metabolised	0.35	-	0.45
xx115	Resistance index	-	0.91	0.96
	TSR allele prop^n	-	0.34	0.31
	ACCase metabolised	-	0.41	0.56
xx125	Resistance index	-	0.99	-
	TSR allele prop^n	-	0.84	-
	ACCase metabolised	-	0.42	-
xx126	Resistance index	0.98	0.90	0.93
	TSR allele prop^n	0.90	0.77	0.75
	ACCase metabolised	0.39	0.48	0.53
xx133	Resistance index	-	0.82	0.97
	TSR allele prop^n	-	0.28	0.50
	ACCase metabolised	-	0.38	0.49
xx135	Resistance index	0.35	0.69	0.79
	TSR allele prop^n	0.06	0.34	0.31
	ACCase metabolised	0.34	0.33	0.45

Table 3.23: Frequency of ACCase target-site alleles. For each year (2012, 2013, 2014), the allele frequency for each mutation has been calculated. Significant differences ($P < 0.05$) with a Fisher's exact test are indicated as: ( ) = no significant difference, (*) = 2013 is significant different from both 2012 and 2014, (**) = 2014 is significant different from both 2012 and 2013.

Mutation	Resistance allele frequency		
	2012	2013	2014
Ile-1781-Leu	0.247	0.404*	0.255
Ile-1781-Val	0	0	0.063**
Ile-1781-Thr	0.007	0.004	0.046**
Trp-2027-Cys	0.004	0.006	0.010
Ile-2041-Asn	0.018	0.027	0.022
Ile-2041-Val	0	0.006*	0
Asp-2078-Gly	0.029	0.025	0.017
Gly-2096-Ala	0.022	0.017	0.017
Total	0.324	0.460	0.348

3.4.4 Trends in management between 2007 and 2014

For the thirteen populations for which field management histories had been provided by the farmer (Table 3.1 – 3.17), the trends in the proportions of each of the eight management practices – winter wheat, late winter wheat sowing, spring cropping, ploughing, glyphosate, pre-emergence herbicide, and post-emergence herbicide - applied was assessed (Figure 3.6). Of thirteen populations, 57% of fields across the 8-years period (2007-2014) sowed winter wheat, peaking with 11/13 populations sowing winter in 2008, and only 3/13 populations sowing winter in 2013 (Figure 3.6a). Of the crops sown, only 5% were late sown (after October 20th) (Figure 3.6b). 14% of crops were spring crops, with years 2011 and 2013 showing peaks in spring cropping of 38% (Figure 3.6c); similarly, 38% of fields in 2011 and 2013 were ploughed – this figure is above the average of 19% of fields ploughed across the eight-year period (Figure 3.6d). 44% of fields applied glyphosate over the eight-year period, with more fields applying glyphosate in the later four-years (2010-2014) (Figure 3.6e). 78% of fields applied at least one pre-emergence herbicide, although

large variation was observed between individual years (Figure 3.6f). More fields applied ALS herbicide (51%, Figure 3.6f) over the eight-year period compare to ACCase herbicides (29%, Figure 3.6g).

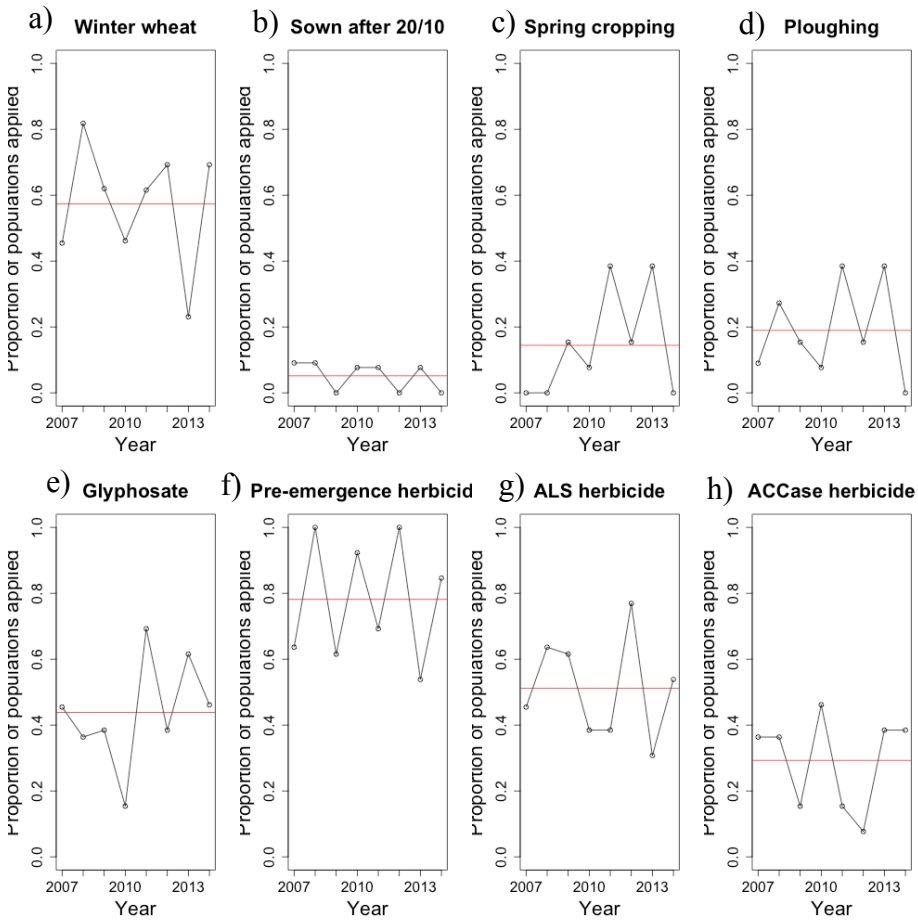


Figure 3.6: Changes in the proportions of eight management factors applied between 2007 and 2014. Management factors: (a) winter wheat, (b) crops sown after 20th October (20/10), (c) spring cropping, (d) ploughing, (e) glyphosate applied, (f) pre-emergence herbicide applied, (g) ALS herbicide applied, and (h) ACCase herbicide applied. For each management factor, the red line indicates the average application of the management factor across all years (2007-2014).

3.5 Discussion

Seventeen populations from a survey conducted in 2011, chosen for their contrasting frequencies of phenotypic resistance, and target-site and enhanced metabolism mechanisms to ALS and ACCase MOA, were re-sampled in 2012, 2013, and 2014. The frequencies of phenotypic resistance, target-site resistance and enhanced

metabolism in each population/year were estimated, to describe how ALS and ACCase have evolved in these *A. myosuroides* populations over this period.

3.5.1 Change in ALS resistance

Across all seventeen populations, the ALS resistance index decreased between 2012 and 2013 (with all but two populations exhibiting no change or a decrease in their ALS resistance index), before increasing in 2014 (for all but one population). The level of ALS TSR in the majority of populations decreased or remained the same between 2012 and 2013, before all but one increased in ALS TSR frequency between 2013 and 2014. Conversely, the average amount of ALS herbicide (mesosulfuron) metabolized decreased - significantly so in 2014 when compared to the previous two years. Although a number of populations expressed a slight increase in mesosulfuron metabolization between 2012 and 2013, and 2013 and 2014 respectively, all but one population exhibiting a decrease in mesosulfuron metabolization between 2012 and 2014. These results suggest that target-site resistance might confer increases in ALS resistance within these populations more than enhanced metabolism. To fully understand the trends in ALS phenotypic resistance, TSR allele frequency and fenoxaprop metabolism in each individual population and across all seventeen populations, they need to be evaluated in light of the weed management that has been applied (see chapter 4).

Pro-197-Thr mutations were identified in greater frequency than Trp-574-Leu mutations in all years, although both mutations appear to be increasing at the same rate (2012-2014). The effect of these target-site mutations on ALS enzyme activity may be an explanation to why Pro-197-Thr mutations are more frequent. Li *et al*

(2012) studied the activity of ALS target-site mutations in *Raphanus raphanistrum* (wild radish), identifying an increase in extractable ALS activity for Pro-197-Ser mutations, and a decrease in extractable ALS activity for Trp-574-Leu mutations. If Pro-197-Thr and Trp-574-Leu mutations in *A. myosuroides* similarly express an increase and decrease in extractable ALS activity respectively, then the increase in pleiotropic fitness associated with the Pro-197-Thr mutation could lead to the mutation being present in higher frequencies.

The overall decrease in ALS enhanced metabolism observed between 2012 and 2014 may indirectly result from the increase in target-site resistance. With higher doses of mesosulfuron-methyl + iodosulfuron-methyl-sodium selecting for target-site mutations over a quantitative trait such as enhanced metabolism (as enhanced metabolic resistance is typically, but not always, low level and dose-dependent) (Délye 2012), an individual possessing an ALS target-site resistance mutation may not necessarily possess genes that endow enhanced metabolism; therefore increases in ALS target-site resistance may spread genes that do not confer enhanced metabolism, leading to the decrease observed.

3.5.2 Change in ACCase resistance

The ACCase resistance index increased between 2012 and 2014, whereas the frequency of ACCase target-site resistance increased between 2012 and 2013, before decreasing again in 2014 to a level similar to that of 2012. The average amount of ACCase herbicide (fenoxaprop) metabolized increased year on year. With a reduced number of fields applying ACCase inhibitors when compared with the number applying ALS inhibitors within the fields studied, though ACCase resistance

remaining high, this may be an indication that there is no fitness cost associated with ACCase resistance and resistance mechanisms. As with ALS resistance, to fully understand the trends in ACCase phenotypic resistance, TSR allele frequency and mesosulfuron metabolism in each individual population and across all seventeen populations, they need to be evaluated in light of the weed management that has been applied (see chapter 4).

A small number of studies have investigated fitness costs of ACCase target-site resistance mutations in *A. myosuroides*: Ile-2041-Asn mutations have been found to confer no fitness cost on plant biomass, height, seed production or germination rate when compared to wildtype individuals (Menchari *et al* 2008; Délye *et al* 2013); Ile-1781-Leu mutations have been found to confer no fitness cost on plant biomass, height or seed production, but delayed germination rates when compared to wildtype individuals (Menchari *et al* 2008; Délye *et al* 2013); but fitness costs in the form of reduced plant biomass, plant height, seed production and accelerated rates of germination have been identified for Asp-2078-Gly mutations (Menchari *et al* 2008; Délye *et al* 2013). The pleiotropic fitness costs of ACCase target-site mutations and enhanced metabolism have been studied in *Lolium rigidum,* finding that individuals possessing target-site resistance exhibited later germination (Vila-Auib *et al* 2005) and ACCase enhanced metabolism reduced vegetative growth and reproductive output by 30% and 23% respectively (Vila-Auib *et al* 2009).

These finding may explain why Ile-1781-Leu mutations are most frequently identified, why Asp-2078-Gly mutations decrease in frequency between 2012 and 2014, and indicate that the low frequency of ACCase inhibitors still being applied is

enough to continue the selection of ACCase enhanced metabolism in light of the potential pleiotropic fitness cost that have been identified in other species.

3.5.3 Importance of management on ALS and ACCase resistance

An integrated weed management (IWM) approach - including spring cropping, ploughing and delayed drilling – has been identified as important in reducing *A. myosuroides* infestations, and are frequently recommended to farmers in order to manage their *A. myosuroides* and resistance issues (Lutman *et al* 2013). Of these three techniques, spring cropping and ploughing appear to have been adopted in greater frequencies in 2011 and 2013. This might be a sign that farmers are heeding this advice and incorporating these practices into their regular management regimes (even though the sowing of winter wheat crops and applications of ALS inhibitors remain frequent.

A number of studies have investigated the presence and extent of ACCase (Moss and Perryman 2007; Délye *et al* 2007; Délye *et al* 2010; Hess *et al* 2012; Hull *et al* 2014; Keshtkar *et al* 2015) and ALS (Chauvel *et al* 2006; Petersen 2011; Hess *et al* 2012) resistance and resistance mechanism in *A. myosuroides* in northern Europe. Similarly, the extent of ACCase and ALS resistance in Australian populations of *Lolium rigidum* has been investigated (Owen *et al* 2014; Malone *et al* 2013). However, these studies have not sampled from the same populations over multiple generations, and most do not take into account the management applied to the field when sampling. The variability between years within population's observed here most likely results from the management applied in the year of sampling. Ideally, samples should be collected from *A. myosuroides* populations not treated with the

herbicide of interest in the year of sampling (as discussed in section 2.5.3) when accurately discerning the presence and frequency of resistance. Therefore, the management applied in the year of sampling should be taken into account when conducting surveys to determine the frequency of resistance within a region.

3.6 Conclusions

The seventeen UK populations studied between 2012 and 2014 exhibit a wide range of changes in phenotypic, target-site and enhanced metabolic resistance. This chapter is purely descriptive of the populations studied. The seventeen UK populations studied between 2012 and 2014 exhibit a wide range of changes in phenotypic, target-site and enhanced metabolic resistance. To understand what causes these changes, subsequent epidemiological analysis (Chapter 4) is needed to determine how the frequency of ALS and ACCase resistance changes in relation to weed management.

4.0 Analyzing the frequency of ACCase and ALS herbicide resistance and resistance mechanisms in UK populations of *Alopecurus myosuroides* in relation to management history.

4.1 Introduction

4.1.1 Epidemiological studies

The epidemiological method, the application of knowledge from studies into the distribution and causes of conditions detrimental to human health (Last 2001), has proven to be an essential tool outside of the field of human health when investigating the distribution and underlying causes of antibiotic, insecticide, fungicide and herbicide resistance evolution (as discussed in section 3.1) (Zhan et al 2008; Hawkey and Jones 2009; Koella *et al* 2009; Pfaller 2012; Evans *et al* 2015). The study by Koella *et al* (2009) is a prime example of using the epidemiological method to investigate and suggest sustainable preventative solutions to a resistance problem. By studying the epidemiology of the *Anopheles* mosquito, a vector of malaria, it was suggested that - as most mosquitoes die before the parasite fully develops, and young mosquitos experience the strongest evolutionary pressure - the use of a larvicide and late-acting insecticide in combination may delay the resistance evolution to the late-acting insecticide (Koella *et al* 2009).

At present, only one published study into herbicide resistance evolution has adopted an epidemiological approach in an attempt to gain such insights. Evans *et al* (2015) studied the frequency of glyphosate resistant *Amaranthus tuberculatus* in 105 fields from Illinois (USA) against soil, landscape, and historical farm management data,

identifying populations with frequent glyphosate applications, high glyphosate doses, and lack of mode of action diversity within a cropping year as exhibiting the greatest frequencies of glyphosate resistant *A. tuberculatus* (Evans *et al* 2015). Epidemiological studies that monitor the change in resistance over a number of generations in relation to management are needed in the case of *A. myosuroides*, so that more effective *A. myosuroides* resistance control strategies can be developed.

4.1.2 Evolutionary and Ecological Models

Ecological systems are highly variable and complex (Jørgensen and Fath 2011). Whether observing a specific trait, biotic/abiotic response, or community interaction, phenotypic variation is an effect of a population's heritable genotypic variation (G), prevailing environmental conditions (E), and genetic*environmental (G*E) interactions (Mayhew 2006). This variability can be a hindrance to our understanding of key ecological and evolutionary processes, in predicting species abundance and compositional change, and developing environmental management regimes, e.g. for species conservation. One way in which we can understand ecological and evolutionary processes more clearly, is through the use of mathematical models. Mathematical models that specifically model evolutionary and ecological processes are abstract, stripping away any unnecessary aspects of the biological system, so that only factors essential to the question being addressed remain (Renton *et al* 2014). Models of this nature have been used to simplify many systems, including Soay sheep population crashes (Coulson *et al* 2001), marine turtle hatching (Hamann *et al* 2011), and honeybee colony population dynamics (Russell *et al* 2013). Models can be of key importance in deciphering processes that drive ecological and evolutionary change, pointing out flaws in our ideas and understanding, generating and testing

predictions or hypothesis, and developing new ideas and ways of thinking (Otto and Day 2007). With these applications in mind, ecological and evolutionary models are extremely useful in agricultural systems, predicting how these systems will change over time in relation to human inputs - the evolution of herbicide resistance in arable weeds is a prime example of this.

4.1.3 Why model herbicide resistance evolution?

The evolution of resistance to herbicides occurs over large temporal and spatial scales, making detailed long-term studies into resistance evolution impractical to conduct (Renton *et al* 2014). This makes simulation models, parameterised and validated by small accompanying experiments, a vitally important tool in identifying and understanding the biological, evolutionary, and ecological components that drive selection for herbicide resistance. Simulation modelling not only allows for important ecological and evolutionary processes underpinning resistance to be determined, but also facilitates the development and evaluation of prospective sustainable chemical and integrated weed management strategies (Renton 2014).

4.1.4 Evolutionary herbicide resistance models

There have been a number of models produced to address a range of questions related to the evolution of herbicide resistance in agricultural weeds. Models have been produced that investigate the evolution of resistance to a certain herbicide MOA, for example, to the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) inhibiting herbicide glyphosate. These glyphosate resistance models include those that focus on the more general operational, genetic, biological and ecological factors that affect glyphosate resistance evolution (Neve 2008), as well as the evolution of

glyphosate resistance in a specific species, e.g. *Lolium rigidum* (Neve *et al* 2003a, 2003b) and *Amaranthus palmeri* (Neve *et al* 2010).

A number of models have been designed that specifically examine the evolution of specific single gene target-site mutations (Richter *et al* 2002; Neve 2008; Jacquemin *et al* 2009; Neve *et al* 2010; Richter *et al* 2012), as well as multi-trait models that explore the evolution of monogenic resistance to multiple MOA (Diggle *et al* 2003; Neve *et al* 2003a, 2003b; Bagavathiannan *et al* 2014). A small number of models have been produced that look at the evolution of the non target-site resistance mechanism of enhanced metabolism (Richter *et al* 2012; Langemann *et al* 2013), and only two have been published that include both mechanisms of target-site and enhanced metabolism (Gardner *et al* 1998; Renton *et al* 2011). Models of herbicide resistance evolution that specifically aim to establish the effect of herbicides used in various combinations (Diggle *et al* 2003), reduced herbicide application rate (Renton *et al* 2011), environmental heterogeneity of herbicide application (Richter *et al* 2002), and spatio-temporal dynamics of herbicide use (Jacquemin *et al* 2009; Richter *et al* 2012), can also be found in the literature.

4.1.5 A. myosuroides herbicide resistance models

A number of the modelling frameworks mentioned above could be applied to *A. myosuroides*. However, to date there has only been a small number of models published that incorporate *A. myosuroides'* biological parameters to investigate herbicide resistance evolution. Models of *A. myosuroides* biology (germination, emergence, etc.) (Colbach and Sache 2001; Colbach *et al* 2006a,b) and the effects of cultivation on seed distribution in the soil (Cousens and Moss 1990) have been

published. Only one model currently exists that investigates the effects of herbicide strategy and cultivation on resistance evolution in *A. myosuroides* (Cavan *et al* 2000). Therefore, models that examine the evolution of resistance in *A. myosuroides*, incorporating multiple MOA, multiple mechanisms of resistance, and certain ecological and evolutionary drivers of resistance are lacking. To improve understanding of how herbicide resistant *A. myosuroides* populations evolve, and how they can be sustainably managed in the UK, a model that combines both target-site and enhanced metabolic resistance to essential ALS and ACCase MOA is needed.

4.2 Objectives

In chapter 3, data sets were presented that monitor the change in phenotypic and genotypic frequencies of ACCase and ALS resistance in a number of *A. myosuroides* populations. For each population, detailed field management histories for the period 2007-2014 were collected. In chapter 4, epidemiological approaches and simulation modelling are used to relate the initial resistance status of populations (2011) and changes in the frequency of resistance and resistance mechanisms (2012-2014) to field management factors. Key questions that are addressed include:

1. Which management factors are associated with high and low frequencies of resistance (epidemiological analysis)?
2. Does real-time (current year) management data enable changes in the frequency of resistance to be predicted (epidemiological analysis)?

3. Does knowledge of field management histories enable simulation models to predict the relative risks of herbicide resistance evolution (simulation modelling)?

4.3 Materials and methods

4.3.1 Epidemiological analysis of resistance in A. myosuroides populations (Q1)

4.3.1.1 Calculating an index for enhanced metabolism (EMR)

The percentage of herbicide metabolised identified with HPLC after 16 hours of incubation allowed each plant to be classified as low metabolism (EMR_{L} = 0-20% of herbicide metabolised), intermediate metabolism (EMR_{I} = 21-50% of herbicide metabolised), or resistant metabolism (EMR_{R} = 51-100% of herbicide metabolised). To calculate an EMR index for each population's annual sample, the number of plants (n) in each of the three metabolism categories ($nEMR_{L}$, $nEMR_{I}$, $nEMR_{H}$) was counted, before multiplying by 0, 0.5 and 1 respectively, to account for individuals in the EMR_{H} group exhibiting a greater degree of EMR than those in the EMR_{I} group, and the $nEMR_{L}$ group having little effect (Equation 4.1). $nEMR_{L}$, $nEMR_{I}$, and $nEMR_{H}$ were summed together before dividing by the total plants tested in each population (n_{total}=16) (Equation 4.1). An EMR index of 0 indicates that no individuals in the population could metabolise herbicide, an EMR index of 1 indicates that all individuals in the population could metabolise a high proportion of the herbicide.

$$\text{EMR index} = \frac{((nEMR_{L}*0) + (nEMR_{I}*0.5) + (nEMR_{H}*1))}{16}$$

Equation 4.1

4.3.1.2 Effect of historical management on resistance and resistance mechanisms

Generalized linear models (glm) were used to investigate the impact of historical management on the frequencies of ALS and ACCase resistance (calculated in section 3.3.5.1), TSR allele frequency (calculated in section 3.3.5.2) and EMR index (calculated in section 4.3.1.1) in the statistical analysis programme R (R Core Development Team 2012).

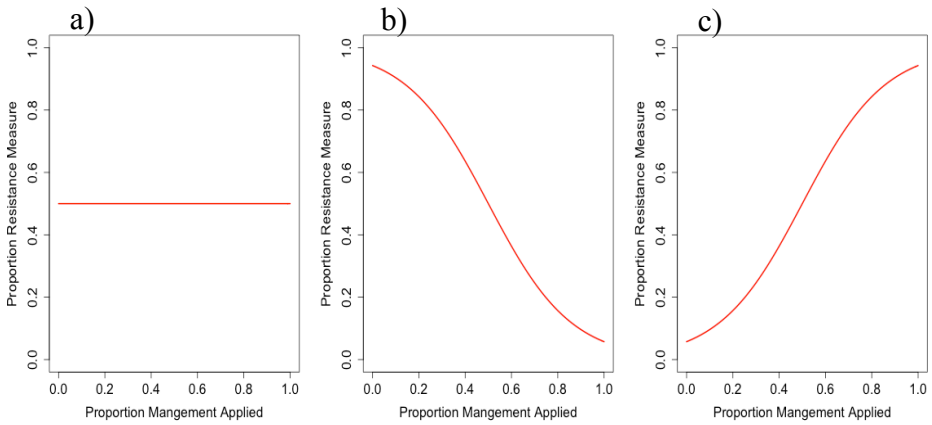


Figure 4.1: Expected correlations produced from the generalized model of proportion of management applied between 2007 and the latest year of sampling in which the MOA of interest was not applied and the measure of resistance (resistance, TSR, or EMR) in the latest year of sampling in which the MOA of interest was not applied. The red line on each graph represents relationship between proportion of the proportion management applied and the measure of resistance when (a) there is no correlation (i.e. the null hypothesis), (b) there is a negative correlation, and (c) there is a positive correlation.

The response variable - estimates of ALS and ACCase resistance, TSR allele frequency and EMR index from the latest year of sampling in which the MOA of interest (either ALS or ACCase) was not applied – were individually modelled against eight explanatory management variables: proportion of years with winter wheat crop sown, winter wheat sowing date as a proportion of the year after October 1st proportion of years with a spring crop sown, proportion of years ploughed, proportion of years when glyphosate applied, proportion of years with a pre-emergence herbicide applied, proportion of years when an ALS herbicide applied,

and proportion of years with an ACCase herbicide applied. This analysis was designed to test the null hypothesis that each management factor has no explanatory power over the response variable (i.e. estimate of ALS and ACCase resistance, TSR allele frequency and EMR index from the latest year of sampling) (Figure 4.1).

4.3.2 Effect of management on the levels of ALS and ACCase resistance between adjacent years (Q2)

In order to establish how the management applied in a single year impacted upon the frequencies of phenotypic resistance, TSR and EMR mechanisms, generalized linear models were fitted with resistance in year t+1 (current year) as the response variable and resistance in year t (previous year) and individual management variables as explanatory variables. Six management factors from the sampling year t+1 – crop sown, sowing period, cultivation, glyphosate application, pre-emergence herbicide application, and post-emergence herbicide application – were divided into discrete categories (e.g. crop sown was divided into winter wheat and non-winter wheat crops (Table 4.1)). Different models were fitted according to the different levels of the management factors and that only data for year t and year t+1 where those management factors were practiced were included in the models (see Figure 4.2 as an example). The null hypothesis of this analysis is that management has no effect on the relationship between t and t+1 observed.

Table 4.1: Management data categories

Management Factor	Categories		
Crop sown	Winter wheat	Non-winter wheat	-
Sowing period	Before 1st Oct	After1st Oct	Spring
Cultivation	Min til	Plough	-
Glyphosate applied	Applied	Not applied	-
Pre-emergent herbicide applied	Applied	Not applied	-
Post-emergent herbicide applied	ALS	ACCase	None

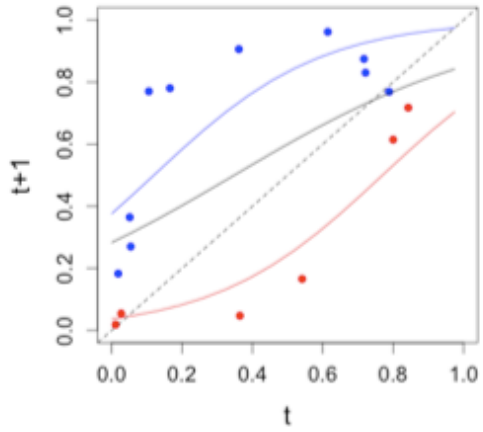


Figure 4.2: Example of analysis for the effect of management on expected correlations produced from the generalized linear comparing resistance measures (resistance, TSR EMR) in sampling year t and t+1. The x-axis indicates the level of resistance, TSR or EMR in year t; the y-axis indicates the level of resistance in year t+1. The dashed line indicates x=y, where resistance in year t+1 = resistance in year t. The data points indicate the relationship between resistance in year t and year t+1 for the sampled populations. The blue and red points represent populations in which a specific management practice has been applied (e.g. ploughing = red, min til= blue). Points above the x=y line represent populations where resistance has increased in year t+1, those below the x=y line represent populations where resistance has decreased. The lines on the graphs are fitted glms. The solid black line fits all data points without considering management. Where the intercept of that line > 0, resistance has increased in year t+1. For the red and blue lines (which relate to certain types of management and the slopes are constrained = the same), where the line is above the solid black line then that management factor causes a greater increase in resistance; where the line is below the black line then that management factor causes a smaller increase in resistance.

4.3.3 Resistance Model (Q3)

4.3.3.1 Model overview

A simulation model of herbicide resistance evolution in *A. myosuroides* was implemented using STELLA visual programming language (version 10.4; iSee systems, NH, USA). The model is based on the life cycle of *A. myosuroides* (Figure 4.3) and follows a similar structure to that of Neve *et al* (2010). The model simulates *A. myosuroides* population dynamics and resistance evolution within a single, discrete population. A population is considered to be all *A. myosuroides* within a single field.

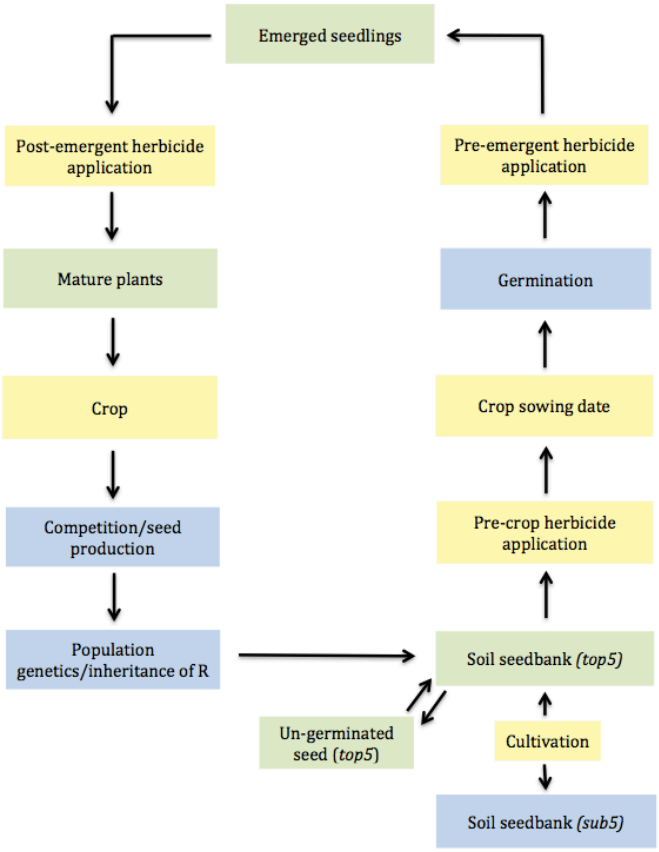


Figure 4.3: Simplified representation of the *A. myosuroides* simulation model. Green boxes the major life-history stages of *A. myosuroides*. The blue boxes are representative of modelling processes that determine the number of individuals that moves from one life-history stage to the next. The yellow boxes are weed management practices applied to the *A. myosuroides* population that affect the number of individuals that moves from one life-history stage to the next. What moves from one life-history stage to the next, is the combination of 21 genotypes created by the combination of ACCase target-site resistance (A), ALS target-site resistance (B), and enhanced metabolism (E) genes in homozygous susceptible (SS), heterozygous resistance (RS), and homozygous resistance (RR) form, as described in section 4.3.3.2. An example of how to interpret the schematic is the number of individual of each genotype that germinates from the soil seedbank (*top5*), is affected by the application of a pre-crop herbicide, and the date in which the crop was sown.

The field size is set at 15ha to represent a typical arable field size in the UK. The initial population size is the product of field size and seed bank density (seeds m^{-2}). The seed bank is comprised of seed resistant (R) and susceptible (S) to ALS and/or ACCase MOA. Uniquely, the model embodies two mechanisms (target-site resistance (TSR) and enhanced metabolism (EMR)) represented in the form of three

single genes: one ACCase target-site resistance gene (A), one ALS target-site resistance gene (B), and one enhanced metabolism endowing resistance to both MOA (E). The initial genotypic proportions of these three mechanisms of resistance within the seedbank are determined by the initial frequency of resistance-conferring alleles. The seed bank is depth structured with seeds present Seedlings germinate and emerge in one of in two layers up to 5cm depth and below 5cm. Seeds are only able to germinate and emerge from the top 5cm of the soil profile. Seeds germinate and emerge in three distinct cohorts, representative of emergence before autumn crop sowing (cohort one), after autumn crop sowing (cohort two), and in spring (cohort three); the survival of R/S plants within each of the three cohorts is then determined by the weed management applied with different emergence cohorts and genotypes being differentially controlled by different management interventions. The amount of seed produced by individuals surviving weed management is calculated using a competition sub-model, and the genotype of new seeds (wildtype (SS), heterozygous (RS), homozygous (RR)) of each resistance genes (A, B, E) is determined in a further sub-model describing the out-crossing breeding of *A. myosuroides*. Finally, the seed produced is returned to the seedbank.

4.3.3.2 Genetic representation of resistance mechanisms

Three single genes - each diallic with Mendelian inheritance - represent ACCase target-site resistance (A), ALS target-site resistance (B), and enhanced metabolism (E) respectively. With the exact genetics of enhanced metabolism (a quantitative trait) unknown, it was therefore not unreasonable to represent enhanced metabolism as a single gene trait for both modes of action. The ACCase target-site resistance gene confers homozygous susceptibility (aa), heterozygous resistance (Aa), and

homozygous resistance (AA) to the ACCase mode of action. The ALS target-site resistance gene confers homozygous susceptibility (bb), heterozygous resistance (Bb), and homozygous resistance (BB) to the ALS mode of action. When the enhanced metabolism gene is heterozygous (Ee) or homozygous (EE) for resistance, it has the capacity to endow enhanced metabolism to one or both MOA depending upon the scenario being tested. Together, the three genes create twenty-one genotypes that are represented throughout the model (Equation 4.1).

$$Genotype = \begin{bmatrix} aabbee & aabbEe & aabbEE & aaBbee & aaBBee & aaBbEe & aaBBEE \\ Aabbee & AabbEe & AabbEE & AaBbee & AaBBee & AaBbEe & AaBBEE \\ AAbbee & AAbbEe & AAbbEE & AABbee & AABBee & AABbEe & AABBEE \end{bmatrix}$$

(Equation 4.1)

4.3.3.3 Management factors considered in the model

Using field history data collected from 17 UK *A. myosuroides* sampled between 2012 and 2014 (described in chapter 3), a number of management factors were identified and included in the model, to assess their effect on resistance evolution. Therefore, the weed management applied in the model consists of eleven crops, two types of cultivation, pre-plant herbicide, and both autumn and spring pre-emergence and post-emergence herbicides. These management factors and the impact they have on the population processes in the model will be discussed throughout sections 4.3.3.4 - 4.3.3.6.

4.3.3.4 A. myosuroides seed bank and seed bank dynamics

4.3.3.4.1 Initial seed population

All model simulations were run within a field that was 15 hectares (150,000m^2) in size, an arable field size typical of that in the UK. At the start of every generation

(model run), the initial seedbank was described by the density of seeds m^{-2}, with an initial density within the top 5cm of the soil profile of 500 seeds m^{-2} chosen to represent a typical *A. myosuroides* infestation in the UK. The initial proportion of wildtype (SS), heterozygous (RS) and homozygous (RR) individuals for each resistance gene within the seed bank populations calculated according to the initial frequency of resistance alleles assuming that the population is in Hardy-Weinberg equilibrium (HWE) (Equation 4.2).

$$p^2*(2pq)*q^2 = 1$$

(Equation 4.2)

Table 4.2: Initial model parameters.

Parameter	Value	Reference
Initial seedbank *top5*	500 seed m^2	
Initial seedbank *sub5*	500 seed m^2	
Field size	150,000m^2 (15ha)	
Germination rate	0.2	Cousens and Moss 1990
Min til	0.2	Cousens and Moss 1990
Ploughing *top5* to *sub5*	0.95	Cousens and Moss 1990
Ploughing *sub5* to *top5*	0.35	Cousens and Moss 1990
Seed mortality	0.5	Cousens and Moss 1990
Cohort emergence	See table 4.3	
Post-emergent herbicide efficacies	See table 4.4	
Pre-emergence herbicide efficacies	See table 4.5	Hull, personal communication
Seeds per seed head	100	Cousens and Moss 1990
Predation factor	0.5	Cousens and Moss 1990
Viability factor	0.55	Cousens and Moss 1990

4.3.3.4.2 Depth structure of seed bank

It is assumed that *A. myosuroides* seed is only able to germinate and emerge from the top 5cm of the soil profile (Naylor 1972), as seed reserves are too small to allow seedlings to grow to the soil surface and emerge from below this depth. Therefore, the seedbank is divided into two distinct layers: the top 5cm level (*top5*) and the sub

5cm level (*sub5*). It is assumed that the initial distribution of seeds between these two layers is uniform so that seeds m^{-2} in *top5* is equal to seeds m^{-2} in *sub5*.

Movement of seed between the *top5* and *sub5* levels of the seedbank is influenced by soil cultivation. Two forms of cultivation are simulated in the model: minimum tillage (assumed to be shallow, non-inversion soil movement of the top 5cm of soil) and ploughing (complete soil inversion to depth 15-20cm). When minimum tillage is practiced, 20% of the seedbank (indiscriminate of genotype) moves from the *top5* to the *sub5* of the seedbank with no upward movement of seed from *sub5* to *top5* (Cousens and Moss 1990). When ploughing is practiced, 0.95 of the seedbank moves from the *top5* to the *sub5* of the seedbank, with 0.35 of the *sub5* seedbank moving to the *top5* seedbank (Cousens and Moss 1990). It is assumed that all movement of seed occurs before sowing of the crop takes place, creating a *top5* and *sub5* seedbank that is "stationary" at *A. myosuroides* emergence (i.e. the seed in the *top5* and *sub5* at the beginning of a generation will remain the same throughout, except for germination of *A. myosuroides* from the *top5* seedbank).

4.3.3.4.3 Annual germination proportion

The proportion of seeds that germinate annually is assumed to be 0.2 (Cousens and Moss 1990). Therefore, as it is assumed that *A. myosuroides* seed is only able to germinate and emerge from the top 5cm of soil - the total annual emergence from the seedbank is calculated by multiplying the number of seeds in the *top5* seedbank by the proportion of seeds that germinate annually.

4.3.3.4.4 Periodicity of seedling emergence

Ninety percent of annual *A. myosuroides* germination occurs in autumn, with a smaller flush of germination in spring (Moss 2013). Within the model, emergence is simulated to occur as three discrete cohorts: Cohort one emerges in autumn before crop sowing, cohort two emerges in autumn following sowing of the crop, and cohort three emerges in spring. The proportion of total emergence that occurs within cohorts has a value related to crop sowing date (Table 4.3) – i.e. with early sowing the size of cohort one is smaller and the size of cohort 2 is bigger. Seed that does not emerge from the *top5* or remains in the *sub5* centimeters of the soil lose viability according to an annual mortality fraction of 0.5.

Table 4.3: Emergence proportion values of cohorts 1 (C1), 2 (C2) and 3 (C3) based on crop sowing date.

Crop Sowing Date	Cohort 1 (C1)	Cohort 2 (C2)	Cohort 3 (C3)
Early autumn - (before 1st Oct)	0.40	0.50	0.10
Mid autumn - (1st to 20th Oct)	0.60	0.30	0.10
Late autumn - (after 20th Oct)	0.70	0.20	0.10
Early spring - (before 15th April)	0.60	0.30	0.10
Late spring - (after 15th April)	0.60	0.35	0.05

4.3.3.5 Herbicide Survival

The weed management applied in the model consists pre-plant, and both autumn and spring pre-emergence and post-emergence herbicides. All pre-emergence herbicides have identical efficacy versus all genotypes (Table 4.5). The efficacy of ALS and ACCase herbicides versus each of the three cohorts depends on application timing and resistance genotype with different resistance mechanisms determining the level

of resistance to ACCase and ALS modes of action applied post-emergent (Table 4.4). Post-emergence herbicides with modes of action other than ACCase and ALS were assumed to provide identical control of all genotypes within a multiplicative survival model (MSM) (Streibig 2003). Where plants possess more than one resistance mechanism that reduces the efficacy of a herbicide, the efficacy is calculated by multiplying the efficacy of TSR by the efficacy of EMR. As an example (taken from Table 4.4), if an individual were to possess heterozygous ALS TSR (ALS herbicide efficacy = 0.05 (5%)) and heterozygous ALS EMR (ALS herbicide efficacy = 0.8 (80%)), the resulting efficacy when an ALS herbicide is applied would be 0.05*0.8 (4%). The number of plants on any single genotype surviving a herbicide application is the product of the number of plants exposed to that herbicide and the herbicide efficacy versus that genotype.

Table 4.4: Efficacies of post-emergent herbicide modes of action for susceptible (sus), heterozygous (het) and homozygous (hom) genotypes. TSR efficacy is the same for both ALS and ACCase MOA. Two different chemical classes of ACCase inhibitor (fops and dims) are considered separately within the model, although it is assumed that ACCase TSR resistance gives identical resistance both. Enhanced metabolism (EMR) gives different levels of resistance to ALS and ACCase (fops and dims); EMR in the model being is most effective against fop, ALS, and dim inhibitors respectively.

Genotype	TSR		EMR		
	ALS	ACCase	Fop	Dim	ALS
Sus	0.99	0.99	0.99	0.99	0.99
Het	0.05	0.05	0.4	0.99	0.8
Hom	0.025	0.025	0.2	0.75	0.4

4.3.3.6 Competition and seed production

The number of *A. myosuroides* seed heads produced per m^2 is calculated using Equation 4.3.

$$((8.71* \text{comp\_plants\_ } m^2)/(1+(0.005741* \text{comp\_plants\_ } m^2))*100)$$

(Equation 4.3)

Table 4.5: Efficacies of all pre-emergence herbicides applied. Pre-emergence herbicide applied = "a + b" = active ingredients applied as separate herbicides, "(a + b)" = active ingredients applied as part of the same herbicide. The pre-emergence herbicide combinations were the various pre-emergence herbicide mixtures used across all years and all populations as identified by the farm management data.

Pre-emergence Combination	Efficacy Estimate
Isoproturon + Trifluralin	0.6
Isoproturon + Pendimethalin	0.5
((Flufenacet + Pendimthalin))	0.7
Simazine + Trifuralin	0.3
Pendimethalin	0.3
Pendimethalin + Diflufenican	0.35
((Flufenacet + Pendimthalin)) + Diflufenican	0.75
Isoproturon + Diflufenican	0.4
Propyzamide	0.9
Triallate + ((Flufenacet + Pendimthalin)) + ((Flufenacet + Diflufenican))	0.95
((Flufenacet + Diflufenican)) +Trifluralin	0.8
((Flufenacet + Diflufenican))	0.7
((Pendimethalin + Imazamox))	0.35
Diflufenican	0.1
((Metzachlor + Propyzamide))	0.95
Trifluralin	0.4
((Flufenacet + Diflufenican)) + Pendimethalin	0.8
((Flufenacet + Diflufenican)) + ((Flufenacet + Flupyrsulfuron))	0.85
Flupyrsulfuron + Flufenacet + Pendimethalin	0.8
((Linuron + Trifluralin)) + ((Flufenacet + Pendimthalin))	0.85
Propyzamide + Trifluralin	0.95
Flupyrsulfuron	0.3
Propyzamide + Carbetamide	0.95
Flupyrsulfuron + ((Flufenacet + Pendimthalin))	0.8
((Metzachlor + Quinmerac + Dimethanamid-p))	0.4
Trifluralin + ((Pendimethalin + Imazamox))	0.6
Trifluralin+((Flufenacet+Pendimthalin))+((Flufenacet+Flupyrsulfuron))	0.9
Metzachlor	0.3
((Flufenacet + Diflufenican)) + Prosulfocarb	0.8
Propyzamide + ((Flufenacet + Diflufenican))	0.95
Prosulfocarb	0.3
((Flufenacet + Diflufenican)) + Flupyrsulfuron	0.8
((Metzachlor + Quinmerac + Dimethanamid-p)) + Propyzmide	0.95
Trifluralin+((Flufenacet+Pendimthalin))	0.8
((Flufenacet+Pendimthalin))+((Flufenacet+Diflufenican))+Prosulfocarb	0.9
Triallate+((Flufenacet + Diflufenican)) + Flupyrsulfuron	0.85

This equation is derived from Cousens and Moss (1990) from field data, (Moss, personal communication). Comp_plants_ m^2 is the total number of surviving plants from the C1, C2 and modified C3 (described below) cohorts that are summed and divided by the field size to get the total number of plants per m^2. The default seed produced per seed head is 100. As cohort 3 (C3) has emerged in spring, plants that emerge in this cohort are less competitive than those emerging in autumn; therefore the seed production capacity of spring emerging (C3) plants is modified by a factor

of 0.75. In effect, each cohort 3 plant is considered as three quarters of a cohort one or cohort two plant. The number of seed heads per m^2 (which has a default value of 100 seed heads per m^2) was modified depending upon the crop sown to account for sowing time and relative competitiveness of different crops. The seed heads per m^2 were modified by 0.75 for spring barley, winter oilseed rape and winter barley because these are sown in spring or spring emerging crops so *A. myosuroides* will produce less seed. Spring beans, spring linseed, spring peas, spring sugar beet, winter linseed, and winter bean crops are modified by 1.25 because these are less competitive than wheat and barley. Spring wheat and winter wheat crops remain at an unmodified 100 seed heads per m^2.

4.3.3.7 Reproduction

A reproduction sub-model describes the inheritance of resistance traits accounting for the breeding system of *A. myosuroides.* Within the model, resistant (R) and susceptible (S) alleles of the three genes - ACCase TSR (A) ALS TSR (B) and enhanced metabolism (E) - are present within the pollen and ova, both of which are haploid. The number of reproductive plants of each genotype is proportional to the number of pollen and ova created. The mutation of gametes (which occurs according to the mutation rate ($1*10^{-6}$)) from susceptible to resistant and resistant to susceptible for the A, B, and E genes is also considered. Finally, gametes are recombined in a random panmictic manner, and mature seeds with the twenty-one resistance genotypes represented (Equation 4.1) are returned to the seed bank. The amount of seed inputted into the seedbank from reproduction (calculated in section 4.3.3.6) is modified before being added to the *top5* seedbank, as a fraction of the seed being returned to the soil will be lost through predation, or through loss of viability

(designated by a predation factor of 0.5 (modified from Moss (1990) and viability factor of 0.55 respectively).

4.3.3.8 Model simulations and initial parameters

4.3.3.8.1 Predicting the relative risks of herbicide resistance evolution

The availability of management data (2007-2011) and knowledge of the resistance status of populations in 2011, presents the opportunity to use the simulation model to explore the resistance risk associated with each field management history and to determine if predicted risks concur with the actual levels of resistance within each population. It was only possible to collect management data for a period five years prior to the initial 2011 survey of populations. The actual resistance status of populations at 2011 will result from management practices prior to 2007 and therefore management practices between 2007 and 2011 were considered indicative of longer-term management approaches for each population. Model simulations were run for a 10-year period, being two iterations of the actual five-year management data collected.

The initial allele frequency of ACCase TSR, ALS TSR and enhanced metabolism was set at 0.01, 0.001 and $1*10^{-6}$ respectively. The spontaneous mutation rate of biological organisms per locus per generation has been cited at approximately $1*10^{-6}$ (Jasieniuk et al 1996). By running the five-year management history twice, the model is essentially being started in 2002 and the initial frequencies that are input into the model are estimated and reflective of this. As ACCase TSR has been selected within *A. myosuroides* populations for some time, the initial frequency of ACCase was considered to be relatively high (0.01). The selection history for ALS TSR may be

more recent, but the initial frequency of resistance is still likely high, but lower than that of ACCase (0.001). With EMR represented as a monogenic trait, it is difficult to assume an initial frequency therefore a value of $1*10^{-6}$ was given.

Enhanced metabolism is a quantitative trait. Within the model however, it has been represented as a monogenic trait as described above. Therefore, in an attempt to more closely replicate the response of EMR in the field, not all individuals possessing a heterozygous or homozygous enhanced metabolism genes were expected to survive selection. Individuals possessing heterozygous and homozygous enhanced metabolism were multiplied by the ACCase and ALS EMR efficacy values respectively from Table 4.4. For example, a population in which 100 of individuals possess a heterozygous ALS EMR mutation predicted by the model, will be multiplied by the heterozygous ALS EMR efficacy value of 0.8 (Table 4.4), so that only 20 individuals remain. To get the value of total resistance within the population, this EMR value added to the proportion of individuals possessing ALS and ACCase TSR and divided by two, to produce a resistance index (RI) in which a value of 0 = no resistance, and 1 = all individuals resistance.

4.3.3.8.2 Real-time management data (current year) predicting changes in the frequency of resistance

This analysis aims to answer the question if the resistance frequency in year t (i.e. 2012 or 2013) and the management in year t+1 (i.e. 2013 or 2014) are known, can the model predict how the resistance frequency will change at the end of year t+1. The resistance frequencies with which the model was initialised were those that were measured in year t, and the management input into the model was that of year t+1. As above, enhanced metabolism is a quantitative trait. Within the model however, it

has been represented as a monogenic trait as described above. Therefore, in an attempt to more closely replicate the response of EMR in the field, not all individuals possessing a heterozygous or homozygous enhanced metabolism genes were expected to survive selection. Individuals possessing heterozygous and homozygous enhanced metabolism were multiplied by the ACCase and ALS EMR efficacy values respectively from Table 4.4.

4.3.3.9 Statistical analysis of modelling data

4.3.3.9.1 Analysing the predicted relative risks of herbicide resistance evolution

For each resistance phenotype, populations were ranked from least to most resistant based on phenotyping of field-collected samples. The management strategies associated with each populations were ranked in terms of resistance risk based on model simulation, with the most high risks strategies being those that predicted the highest frequencies of resistance. The model data and field data was then regressed against each other using a linear model in R (R Development Core Team 2012) to see if there was a significant relationship. Further analysis was conducted on the ALS and ACCase resistance index, proportion of TSR and proportion of EMR of the field and model data. Using a generalized linear model in which the response variable was the field data, and the explanatory variable was the model predictions of the resistance index, proportion of TSR and proportion of EMR was determined. Generalized linear models were also used to compare the effect of the proportion management applied on the field and model data for all response variables. If the effect of management were the same in the model as in the field, the correlation between resistance index, TSR and EMR would be the same for both field data and model predictions.

4.3.3.9.2 Analysis of real-time management data (current year) predicting changes in the frequency of resistance to be predicted.

To assess whether the model could accurately predict the levels of resistance, TSR and EMR in year t+1 after inputting resistance parameters from year t and management data from year t+1, the resistance, TSR and EMR model predictions were compared to the field data using a generalized linear model in which the response variable was the level of resistance, TSR or EMR in year t and the explanatory variable was the level of resistance, TSR and EMR in year t+1 in R (R Development Core Team 2012). An analysis of variance (ANOVA) R (R Development Core Team 2012) was used to compare values of the ALS and ACCase resistance index, proportion of TSR and proportion of EMR between the raw field and model data to identify whether there was a significant difference between them.

4.4 Results

4.4.1 Management affects on resistance

4.4.1.1 Management affects on ALS, resistance, TSR, and EMR

The only statistically significant ($P = 0.037$) relationship identified was a negative relationship between ALS RI and the proportion of spring crops sown between 2007 and latest year of sampling in which an ALS MOA was not applied (Figure 4.4a). ALS TSR (Figure 4.4a) also decreased as the proportion of spring crops sown increased, but this relationship was not statistically significant ($P < 0.2 > 0.05$); ALS EMR (Figure 4.4a) exhibited no statistically significant ($P > 0.2$) relationship to proportion of spring crops sown. The relationship identified between ALS RI and the proportion of years that glyphosate was applied between 2007 and latest year of sampling in which an ALS MOA was not applied was that of a non-significant increase ($P < 0.2 > 0.05$) (Figure 4.4b). ALS EMR (Figure 4.4a) also increased as the

proportion of years that glyphosate was applied increased, but this relationship was not statistically significant (P < 0.2 > 0.05); ALS TSR (Figure 4.4b) exhibited no statistically significant (P > 0.2) relationship to proportion of years that glyphosate was applied. The relationship identified between ALS RI and the proportion of years ploughed between 2007 and latest year of sampling in which an ALS MOA was not applied was that of a non-significant decrease (P < 0.2 > 0.05) (Figure 4.4c); ALS TSR (Figure 4.4c) and EMR (Figure 4.4c) exhibited no statistically significant (P > 0.2) relationship to proportion of years ploughed. All other ALS resistance, TSR and EMR relationships with management - proportion of years with winter wheat crop sown, winter wheat sowing date as a proportion of the year after 15/8, proportion of years with a pre-emergence herbicide applied, proportion of years when an ALS herbicide applied, and proportion of years with an ACCase herbicide applied - were not statistically significant (P > 0.05).

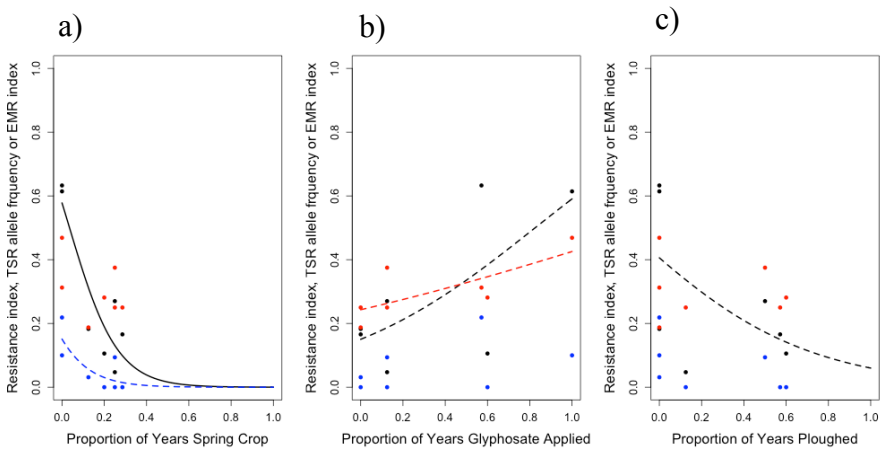


Figure 4.4: Relationships between ALS resistance index (RI) (black), ALS TSR (blue) and possessing ALS EMR (red) and the frequency of management applied: (a) Proportion of years with a spring crop sown, (b) proportion of years when an Glyphosate was applied, and (c) proportion of years ploughed. Solid lines indicate a significant (P < 0.05) relationship between the resistance measure and management. Dashed lines indicate a non-significant (P < 0.2 > 0.05) relationship trend between the resistance measure and management. No line indicates a non-significant (P > 0.2) relationship between the resistance measure and management.

4.4.1.2 Management affects on ACCase, resistance, TSR, and EMR

Of all of the ACCase RI, TSR and EMR correlations, three relationships showed a statistically significant correlation between proportion management factor and resistance measure (Figure 4.5).

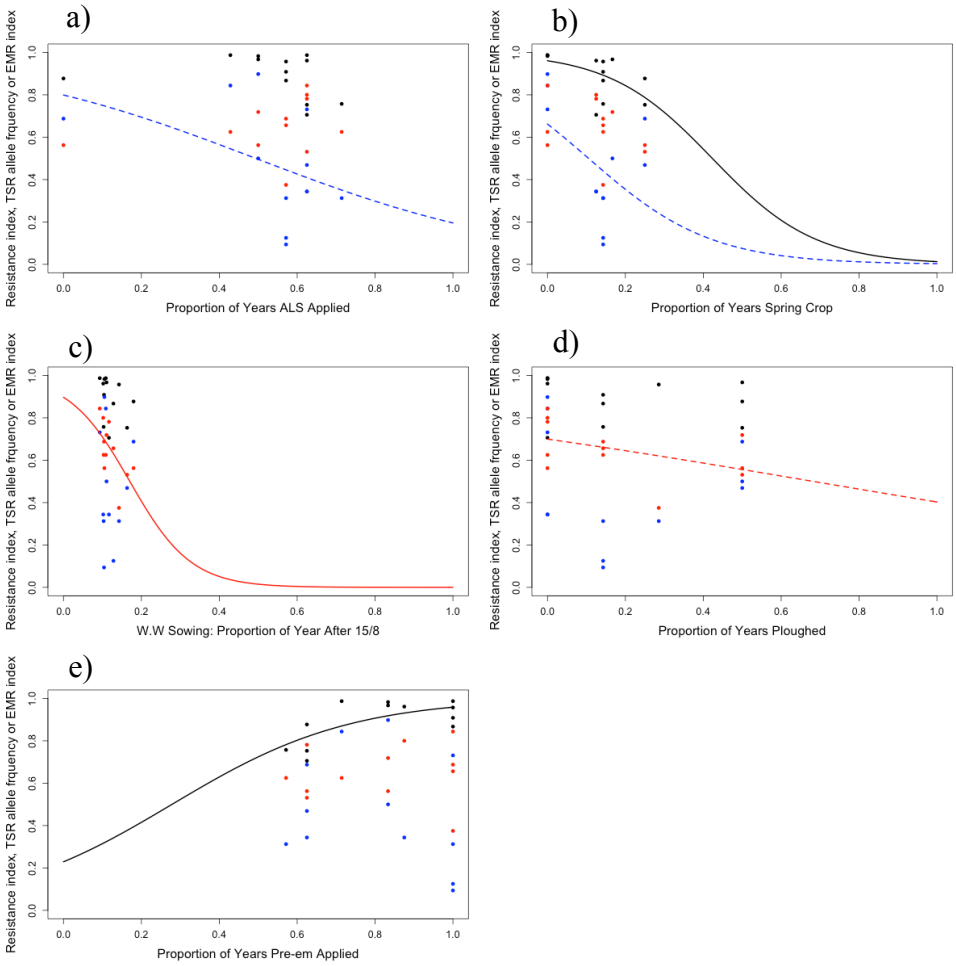


Figure 4.5: Relationships between ACCase resistance index (RI) (black), ACCase TSR (blue) and possessing ACCase EMR (red) and the frequency of management applied: (a) Proportion of years ALS herbicide applied, (b) proportion of years spring crop sown, (c) winter wheat sowing as a proportion of the year after 15/8, (d) proportion of years ploughed, (e) proportion of years pre-emergence herbicide applied. Solid lines indicate a significant ($P < 0.05$) relationship between the resistance measure and management. Dashed lines indicate a non-significant ($P < 0.2 > 0.05$) relationship trend between the resistance measure and management. No line indicates a non-significant ($P > 0.2$) relationship between the resistance measure and management.

Later winter wheat sowing dates exhibiting a significantly lower level of ACCase EMR (P = 0.038) (Figure 4.5c), increased application of pre-emergence herbicide exhibiting higher levels of ACCase resistance (P = 0.026) (Figure 4.5e), and higher amounts of spring cropping exhibiting significantly lower levels of ACCase resistance (P = 0.049) (Figure 4.6b).

Non-significant negative trends were also identified between ACCase TSR and the proportion of years ALS herbicide applied (P = 1.88) (Figure 4.5a), ACCase TSR and the proportion of spring crops applied (P = 0.127) (Figure 4.5b), and ALS EMR and the proportion of years ploughed (P = 0.139) (Figure 4.5d). All other relationships between measures of ACCase resistance and management - proportion of years with winter wheat crop sown, proportion of years glyphosate applied, and proportion of years with an ACCase herbicide applied - were non-significant (P > 0.05).

4.4.2 Effect of management on the proportion of ALS resistant seed returned

4.4.2.1 Change in ALS resistance, TSR and EMR between years

The overall size of the change in ALS resistance, TSR and EMR between adjacent years was quantified using generalized linear models (glm). The trend in ALS resistance between year's t and t+1 is that of an increase, as indicated by the intercept of 0.28 and 11 of the 16 populations falling above the x=y line (Figure 4.6a), with an odds ratio ("slope") of 1.31 being indicative that lower initial values (t) are more likely to increase in year t+1. ALS TSR also exhibited a small increase in frequency in year t+1 when compared to year t (intercept = 0.09, 7 of the 16 populations falling above the line x=y, with an odds ratio of 1.59) (Figure 4.6b). EMR also exhibited an

increase in frequency in year t+1 when compared to year t (intercept = 0.18 and 7 of the 16 populations falling above the line x=y) (Figure 4.6c), with an odds ratio of 1.28 indicative of lower initial values of EMR increasing while higher values remain constant or decrease.

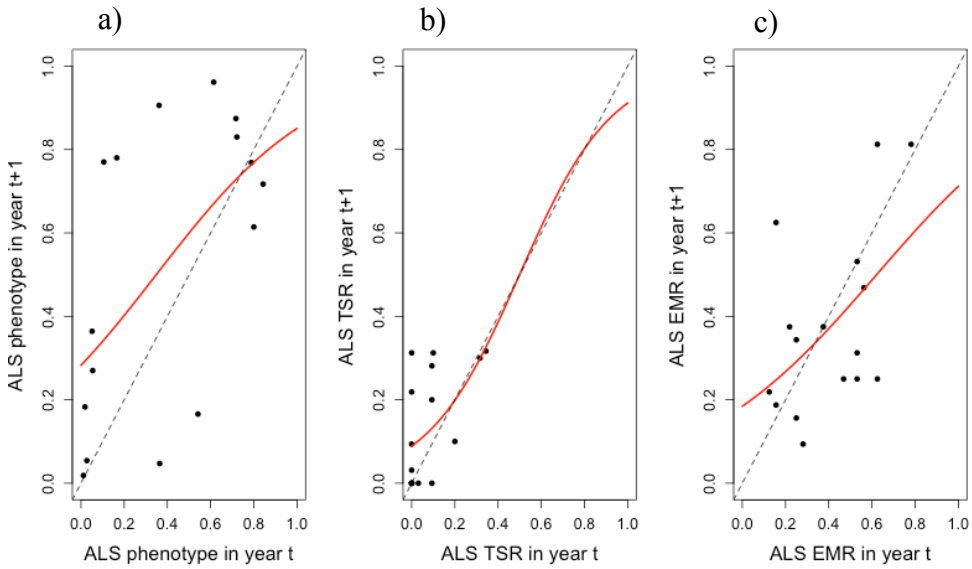


Figure 4.6: The change in (a) ALS resistance, (b) ALS TSR and (c) ALS EMR between year t and t+1. The dashed black line represents no change in resistance between years for comparison. The y-axis intercept is indicative of the increase or decrease in phenotype, TSR or EMR between years. If the line is above 0, this indicates that there has been an increase in the measure of resistance.

4.4.2.2 Effect of management on the change in ALS resistance, TSR and EMR between years

Significantly higher intercepts (Table 4.6) were identified for the sowing of winter wheat, crops sown after 1st October and the application of an ALS herbicide, indicating that populations managed using these factors were associated with significantly higher levels of ALS resistance in year t+1 when compared to year t (Figure 4.7a-c). Cultivation practices and pre-emergence herbicide use did not have a significant ($P < 0.2 > 0.05$) impact on the level of ALS resistance observed in year

t+1, although populations practicing minimum tillage and using a pre-emergence herbicide exhibited larger increases in ALS RI (Figure 4.7d-e). Glyphosate application had no significant effect on the level of ALS RI.

The only significant management effect on the frequency of ALS TSR was identified for the sowing of winter wheat, with winter wheat crops being associated with significantly higher levels of ALS TSR in year t+1 when compared to year t (Table 4.6, Figure 4.8a). Crops sown after 1st October, the application of an ALS herbicide, and glyphosate application did not have a significant ($P < 0.2 > 0.05$) impact on the level of ALS resistance observed in year t+1, although sowing dates after October 20th, application of an ALS herbicide and application of glyphosate exhibited larger increases in ALS TSR (Figure 4.8 b-d). Cultivation and application of a pre-emergence herbicide had no significant effect ($P < 0.05$) on the change in ALS TSR between year t and t+1.

None of the management factors exhibited any significant ($P > 0.05$) impact upon the level of ALS enhanced metabolism between year t and year t+1, although the sowing of winter wheat, application of an ALS herbicide and the sowing of crops after October 20th exhibited larger increases in ALS EMR (Table 4.6, Figure 4.9a-c).

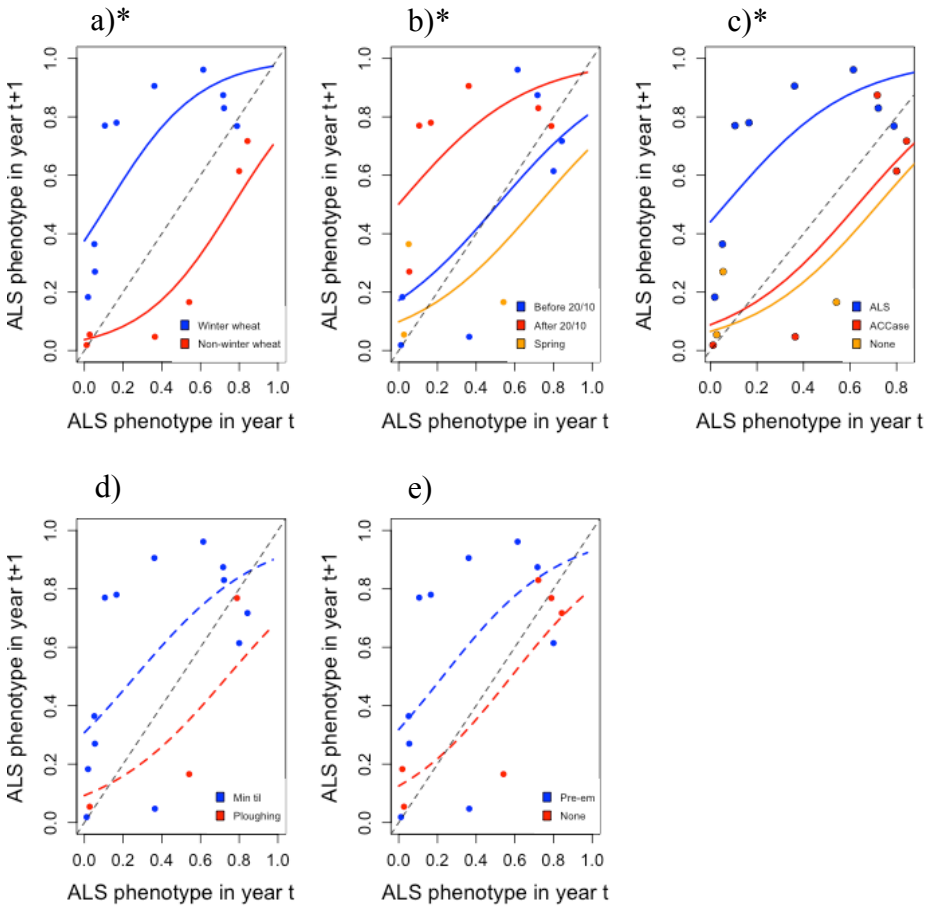


Figure 4.7: Relationship between proportion ALS resistant in the year of sampling (t+1) (y-axis) and the previous sampling year (t) (x-axis), taking into account the affect of management: (a) winter wheat (blue) Vs. non-winter wheat (red) crops, (b) = sowing date: before 1st October, (blue), after 1st October (red), spring (orange), (c) post-emergence herbicide application: ALS (blue), ACCase (red), none (orange), (d) cultivation: minimum tillage (blue), ploughing (red), and (e) pre-emergence herbicide application: pre-em applied (blue), pre-em not applied (red). Graph letters labelled with an asterisk (*) and solid lines indicate a significant ($P < 0.05$) relationship between the resistance measure and management. Dashed lines coloured indicate a non-significant ($P < 0.2 > 0.05$) relationship between the resistance measure and management. The dashed black line represents no change in resistance between years for comparison.

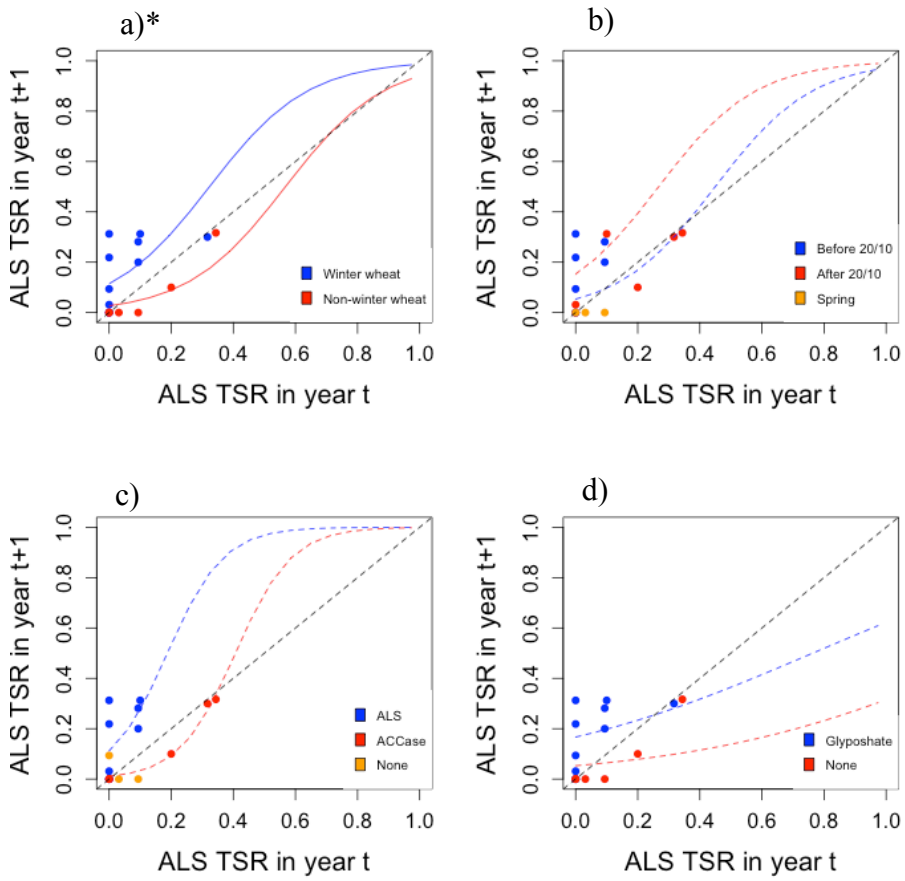


Figure 4.8: Relationship between proportion ALS TSR in the year of sampling (t+1) (y-axis) and the previous sampling year (t) (x-axis), taking into account the affect of management: (a) winter wheat (blue) Vs. non-winter wheat (red) crops, (b) = sowing date: before 1st October, (blue), after 1st October (red), spring (orange), (c) post-emergence herbicide application: ALS (blue), ACCase (red), none (orange), and (d) glyphosate applied (blue) Vs. no-glyphosate applied (red). Graph letters labelled with an asterisk (*) and solid lines indicate a significant ($P < 0.05$) relationship between the resistance measure and management. Dashed lines coloured indicate a non-significant ($P < 0.2 > 0.05$) relationship between the resistance measure and management. The dashed black line represents no change in resistance between years for comparison.

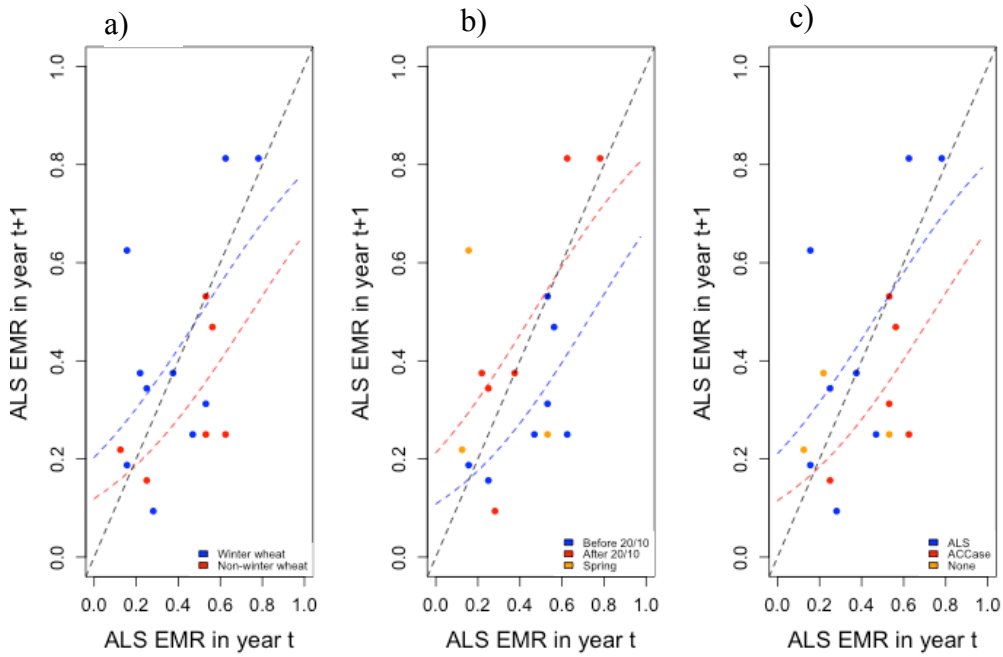


Figure 4.9: Relationship between proportion ALS EMR in the year of sampling (t+1) (y-axis) and the previous sampling year (t) (x-axis), taking into account the affect of management: (a) winter wheat (blue) Vs. non-winter wheat (red) crops, (b) = sowing date: before 1st October, (blue), after 1st October (red), spring (orange), and (c) post-emergence herbicide application: ALS (blue), ACCase (red), none (orange). Graph letters labelled with an asterisk (*) and solid lines indicate a significant ($P < 0.05$) relationship between the resistance measure and management. Dashed lines coloured indicate a non-significant ($P < 0.2 > 0.05$) relationship between the resistance measure and management. The dashed black line represents no change in resistance between years for comparison.

Table 4.6: Estimate of management effects on ALS resistance phenotype, frequency of TSR alleles and enhanced metabolism from general linear models. The intercept and the P-value for each management factor are indicated. P-values marked with two asterisks (**) indicate management practices that have significantly different effects on the level of ALS resistance, TSR and EMR; P-values marked with an asterisk (*) indicate management practices that have a non-significant effect (P < 0.2 > 0.05) on the level of ALS resistance, TSR and EMR; unmarked P-values () indicate management practices that have a no significant effect (P > 0.05) on the level of ALS resistance, TSR and EMR.

Management		Phenotype		Target-site alleles		Enhanced metabolism	
		Intercept	P-value	Intercept	P-value	Intercept	P-value
Crop	Winter Wheat	0.37	0.001**	0.11	0.031**	0.20	0.181*
	Non-wheat	0.037		0.03		0.12	
Sowing	Before 1/10	0.17		0.05		0.11	
	After 1/10	0.50	0.057**	0.15	0.099*	0.21	0.180*
	Spring	0.10		0		0.21	
Cultivation	Min til	0.31	0.129*	0.12	0.996	0.18	0.982
	Plough	0.09		0		0.19	
Glyphosate	Applied	0.86	0.008**	0.17	0.145*	0.02	0.007**
	Not	0.25		0.05		0.36	
Pre-em	Applied	0.32	0.161*	0.10	0.364	0.38	0.033**
	Not	0.13		0.06		0.10	
Post-em	ALS	0.44		0.11		0.21	
	ACCase	0.08	0.020**	0.01	0.063**	0.12	0.186*
	Not	0.07		0.13		0.14	

4.4.3 Effect of management on the proportion of ACCase resistance

4.4.3.1 Change in ACCase resistance, TSR and EMR between years

The overall size of the change in ACCase resistance, TSR and EMR between adjacent years was quantified using generalized linear models (glm). The trend in ACCase resistance between year's t and t+1 is that of an increase, as indicated by the intercept of 0.04 and 10 of the 16 populations falling above the x=y line (Figure 4.10a), with an odds ratio ("slope") of 1.93 indicative that higher initial values (t) are more likely to increase in year t+1. ACCase TSR also exhibited a small increase in frequency in year t+1 when compared to year t (intercept = 0.30 and 8 of the 16 populations falling above the line x=y) (Figure 4.10b), with an odds ratio of 1.26 indicating that lower initial levels (t) of TSR exhibit the greatest increase. EMR exhibited no significant relationship between years t and t+1 – although 11 of the 16 populations fall above the x=y line (Figure 4.10c), therefore the increase in frequency in year t+1 was greatest in populations with lower levels of EMR in year t.

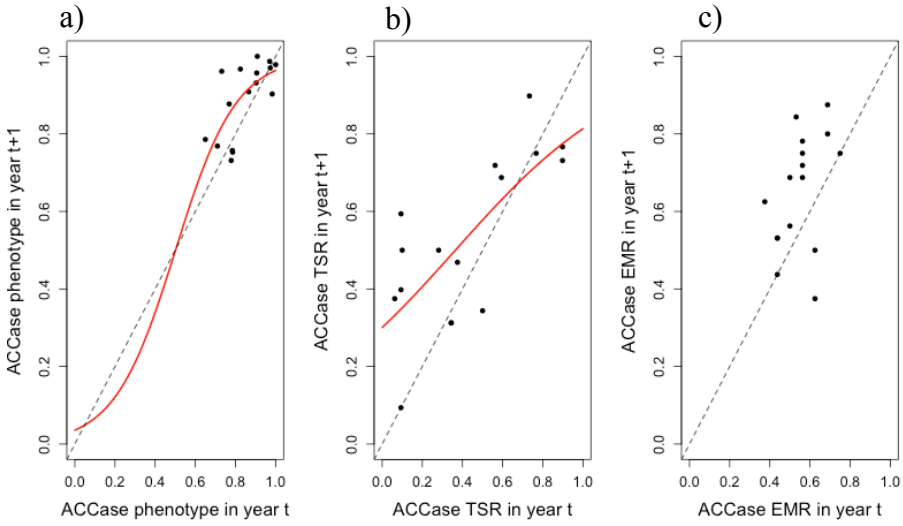


Figure 4.10: The change in (a) ACCase resistance, (b) ACCase TSR and (c) ACCase EMR the current year of sampling (t+1) and previous year of sampling (t). The dashed black line represents no change in resistance between years for comparison. The y-axis intercept is indicative of the increase or decrease in phenotype, TSR or EMR between years. If the line is above 0, this indicates that there has been an increase in the measure of resistance.

4.4.3.2 Effect of management on the change in ACCase resistance, TSR and EMR between years

There was no significant effect of management on the levels (intercept values) of ACCase phenotypic resistance (Table 4.7) meaning that management has no significant effect in the level of resistance observed in section 4.4.3.1.

The only significant management effect on the frequency of ACCase TSR was identified for the sowing of winter wheat and post-emergent herbicide application, with significantly higher levels of ACCase TSR in year t+1 when compared to year t identified in non-winter wheat crops treated with an ACCase herbicide (Table 4.7, Figure 4.11a,c). Cultivation did not have a significant ($P < 0.2 > 0.05$) impact on the level of ALS resistance observed in year t+1, although ploughed fields exhibited larger increases in ALS TSR (Figure 4.11c). Although some variation is observed among the categories of the other three management factors tested – sowing date, glyphosate application and pre-emergence herbicide application - they had no significant effect on the increase in ACCase TSR.

No significant management factors were identified as having a significant effect on the level of ACCase enhanced metabolism, although crops sown before 1st October did have a non-significant ($P < 0.2 > 0.05$) impact on the level of ACCase EMR observed in year t+1,exhibited larger increases in ACCase EMR (Table 4.7, Figure 4.12).

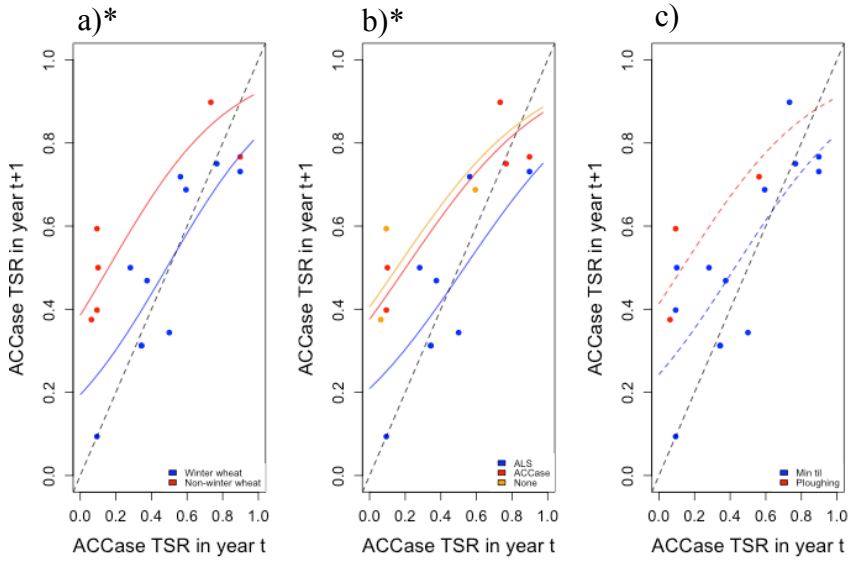


Figure 4.11: Relationship between proportion ACCase TSR in the year of sampling (t+1) (y-axis) and the previous sampling year (t) (x-axis), taking into account the affect of management: (a) winter wheat (blue) Vs. non-winter wheat (red) crops, (b) post-emergence herbicide application: ALS (blue), ACCase (red), none (orange), and (c) cultivation: minimum tillage (blue), ploughing (red). Graph letters labelled with an asterisk (*) and solid lines indicate a significant ($P < 0.05$) relationship between the resistance measure and management. Dashed lines coloured indicate a non-significant ($P < 0.2 > 0.05$) relationship between the resistance measure and management. The dashed black line represents no change in resistance between years for comparison.

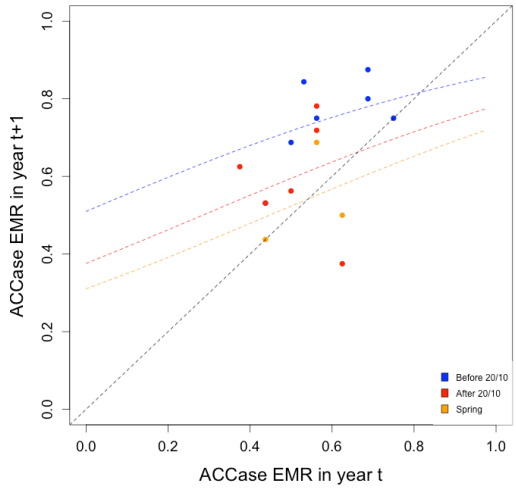


Figure 4.12: Relationship between proportion ACCase EMR in the year of sampling (t+1) (y-axis) and the previous sampling year (t) (x-axis), taking into account the affect of management sowing date: before 1st October, (blue), after 1st October (red), spring (orange). Graph letters labelled with an asterisk (*) and solid lines indicate a significant ($P < 0.05$) relationship between the resistance measure and management. Dashed lines coloured indicate a non-significant ($P < 0.2 > 0.05$) relationship between the resistance measure and management. The dashed black line represents no change in resistance between years for comparison.

Table 4.7: Estimate of management affects on ACCase resistance phenotype, frequency of TSR alleles and enhanced metabolism from general linear models. The intercept and the P-value for each management factor are indicated. P-values marked with two asterisks (**) indicate management practices that have significantly different effects on the level of ALS resistance, TSR and EMR; P-values marked with an asterisk (*) indicate management practices that have a non-significant effect (P < 0.2 > 0.05) on the level of ALS resistance, TSR and EMR; unmarked P-values () indicate management practices that have a no significant effect (P > 0.05) on the level of ALS resistance, TSR and EMR.

Management		Phenotype		Target-site alleles		Enhanced metabolism	
		Intercept	P-value	Intercept	P-value	Intercept	P-value
Crop	Winter Wheat	0.05	0.57	0.19	0.012**	0.31	0.726
	Non-wheat	0.04		0.39		0.29	
Sowing	Before 1/10	0.04	0.590	0.33	0.597	0.51	0.059*
	After 1/10	0.03		0.28		0.38	
	Spring	0.04		0.79		0.31	
Cultivation	Min til	0.02	0.683	0.24	0.087*	0.32	0.334
	Plough	0.03		0.41		0.24	
Glyphosate	Applied	0.03	0.922	0.29	0.940	0.31	0.600
	Not	0.03		0.30		0.36	
Pre-em	Applied	0.03	0.862	0.30	0.941	0.30	0.963
	Not	0.03		0.30		0.30	
Post-em	ALS	0.01	0.675	0.21	0.027**	0.38	0.119
	ACCase	0.01		0.38		0.41	
	Not	0.01		0.41		0.24	

4.4.4 Simulation models to predict ALS resistance risk under different field management scenarios

The resistance index (RI), TSR and EMR indexes predicted in year 10 of the model were compared with RI, TSR and EMR values calculated from the 2011 sampling year (Figure 4.13).

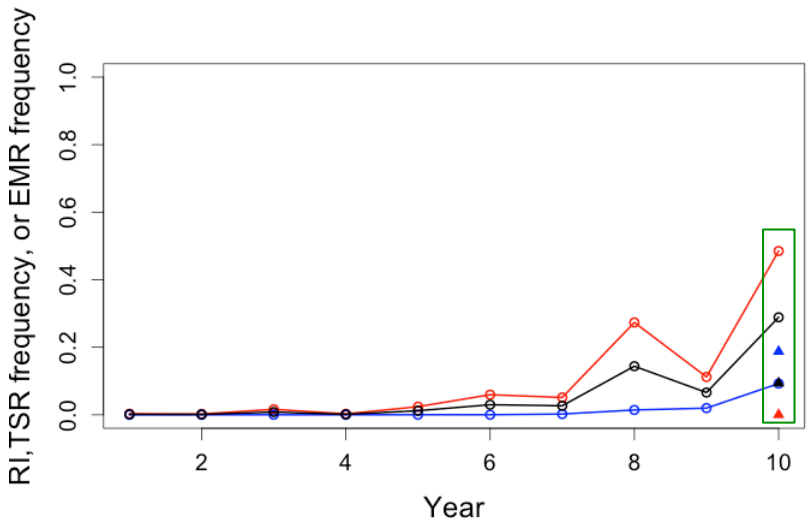


Figure 4.13: Example of ALS model output for population xx066. The modelling output over the ten years for which the model was run for ALS TSR (red line), ALS EMR (blue line) and ALS resistance index (RI) (black line). The model predictions in year 10 (green box) are used to compare with ALS TSR (red triangle), EMR (blue triangle) and RI (black triangle) values calculated for the 2011 year of sampling. The values for ALS TSR, EMR and RI predicted from the model and calculated from field are used in subsequent analysis.

All correlations between model predictions and field data for ALS – model vs. data population rank ($P = 0.64$), model vs. data resistance ($P = 0.44$), model vs. data TSR ($P = 0.54$), model vs. data EMR ($P = 0.56$) - were not significant ($P > 0.05$) (Figure 4.14). For 6 populations the rank predicted by the model was lower than estimated for the field data, while the rank for 7 populations was higher than estimated for the field data (Figure 4.14a).

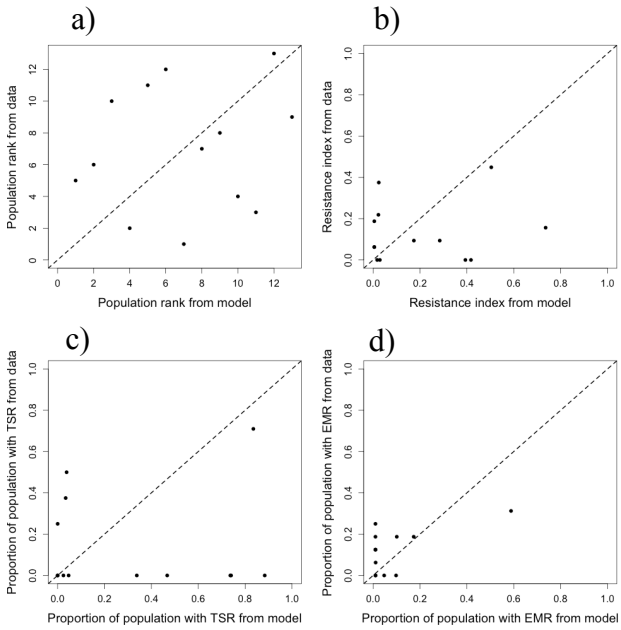


Figure 4.14: Relationship between modelling vales and field data for (a) ALS rank, (b) ALS resistance index, (c) ALS TSR, and (d) ALS EMR. The dashed black line represents no change in resistance between years for comparison.

The proportion of each management factor applied between 2007 and 2011 was calculated, and the relationship between this proportion and the levels of TSR and EMR predicted by the model and identified in field data compared. This approach is used to try and determine if model errors could be attributed to particular parameters relating to some of the management. No significant ($P > 0.05$) effect of management on ALS TSR and EMR was identified for either modelling or field data (Figures 4.15 and 4.16). However in nearly all cases, except for proportion of years spring crop and ACCase applied - the proportion of TSR in the modelling data (blue line) always exhibits a non-significantly ($P < 0.2 > 0.05$) different response to that of TSR from the field data. This is an indication that the effect these parameters have on ALS TSR are either too small, resulting in much higher levels of TSR.

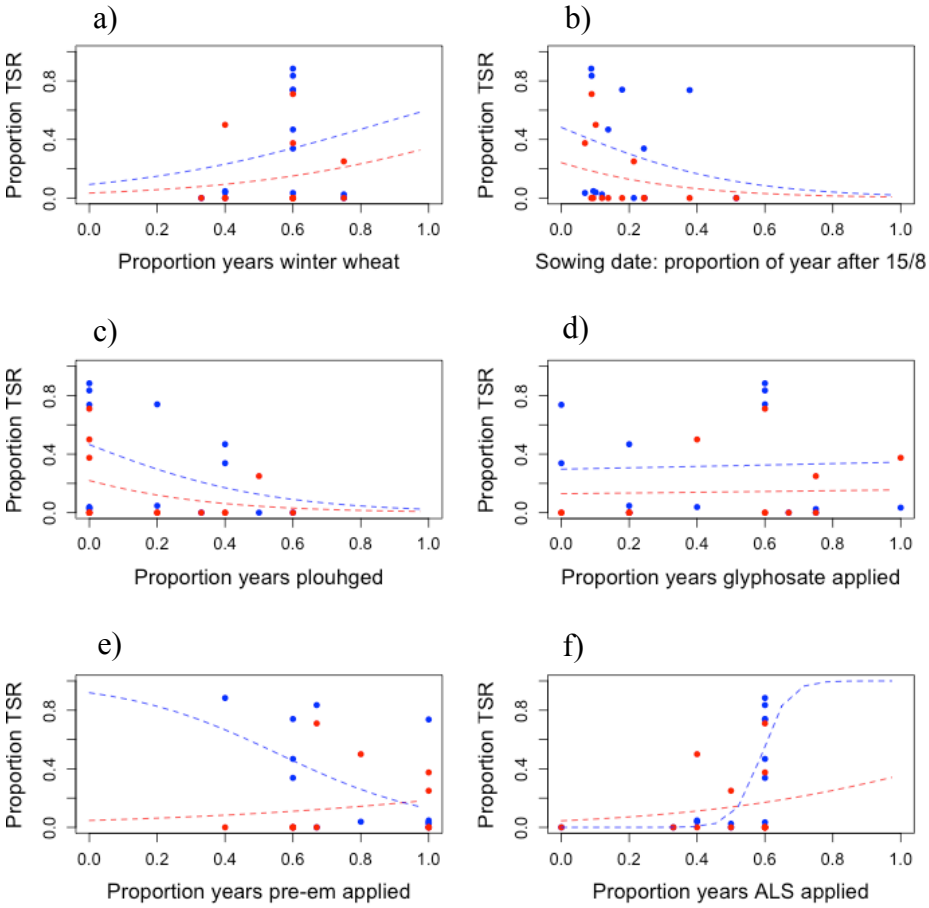


Figure 4.15: Relationships between the proportion of ALS TSR for model (blue) and field (red) data and the frequency of management applied: (a) proportion of years with winter wheat crop sown, (b) winter wheat sowing date as a proportion of the year after 15/8, (c) proportion of years ploughed, (d) proportion of years when an Glyphosate was applied, (e) proportion of years in which a pre-emergence herbicide was applied, and (f) proportion of years when an ALS herbicide was applied. Graph letters labelled with an asterisk (*) indicates a significant relationship of the resistance measure and the proportion management factor. Solid lines indicate a significant ($P < 0.05$) relationship between the resistance measure and management. Dashed lines coloured indicate a non-significant ($P < 0.2 > 0.05$) relationship between the resistance measure and management.

The proportion of EMR in the modelling data (blue line) always exhibits a non-significantly ($P < 0.2 > 0.05$) different response to that of EMR from the field data for ploughing and pre-emergence application (Figure 4.16). This is an indication that the effects these parameters have on ALS EMR are either too large or too small. Other parameters exhibited no significant difference between model and field data.

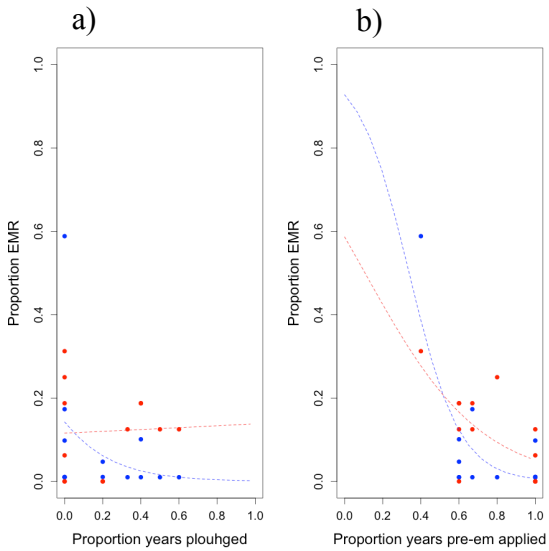


Figure 4.16: Relationships between the proportion of ALS EMR for model (blue) and field (red) data and the frequency of management applied: (a) proportion of years ploughed and (b) proportion of years in which a pre-emergence herbicide was applied. Graph letters labelled with an asterisk (*) indicates a significant relationship of the resistance measure and the proportion management factor. Solid lines indicate a significant ($P < 0.05$) relationship between the resistance measure and management. Dashed lines coloured indicate a non-significant ($P < 0.2 > 0.05$) relationship between the resistance measure and management.

4.4.5 Simulation models to predict ACCase resistance risks under different field management scenarios

All correlations between model predictions and field data for ACCase – model vs. data population rank ($P = 0.48$), model vs. data resistance ($P = 0.66$), model vs. data TSR ($P = 0.90$), model vs. data EMR ($P = 0.06$) - were not significant ($P < 0.05$) (Figure 4.19a). For 7 populations the rank predicted by the model was lower than estimated for the field data, while the rank for 5 populations was higher than estimated for the field data; one of the population had it's rank correctly predicted by the model (Figure 4.17a).

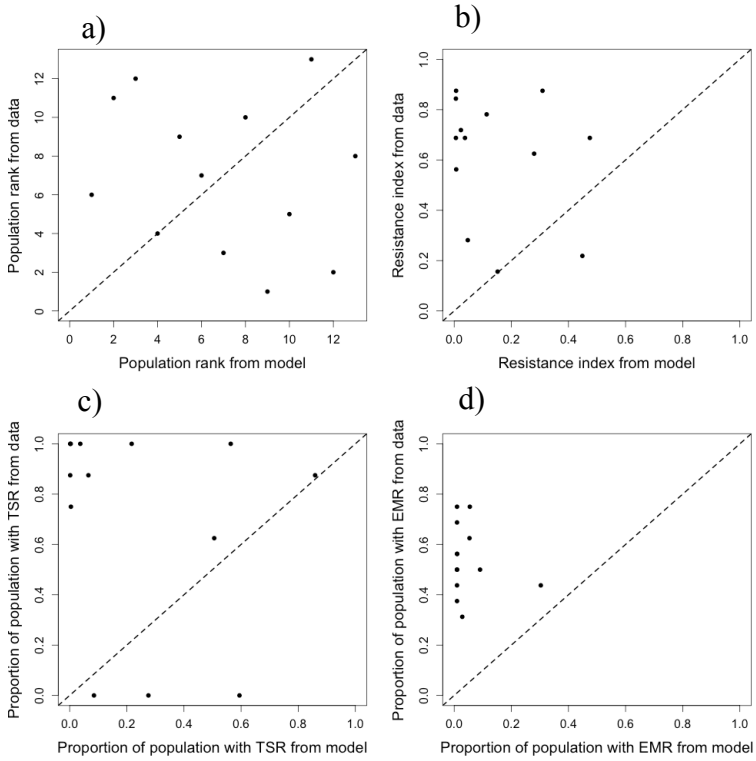


Figure 4.17: Relationship between modelling vales and field data for (a) population rank, (b) ACCase resistance index, (c) ACCase TSR, and (d) ACCASE EMR. The red line indicates the relationship between modelling vales and field data. The dashed black line represents no change in resistance between years for comparison.

The proportion of each management factor applied between 2007 and 2011 was calculated, and the relationship between this proportion and the levels of ACCase resistance, TSR and EMR predicted by the model and identified in field data compared. No significant ($P > 0.05$) effect of management on ACCase TSR and EMR was identified for either modelling or field data. However in nearly all cases, proportion of TSR and EMR in the field data (red line) is always significantly ($P < 0.05$) greater than that of ACCase resistance, TSR or EMR predicted by the model. This is an indication that the effect these parameters have on ACCase TSR and EMR are either too great, resulting in lower levels of ACCase TSR and EMR.

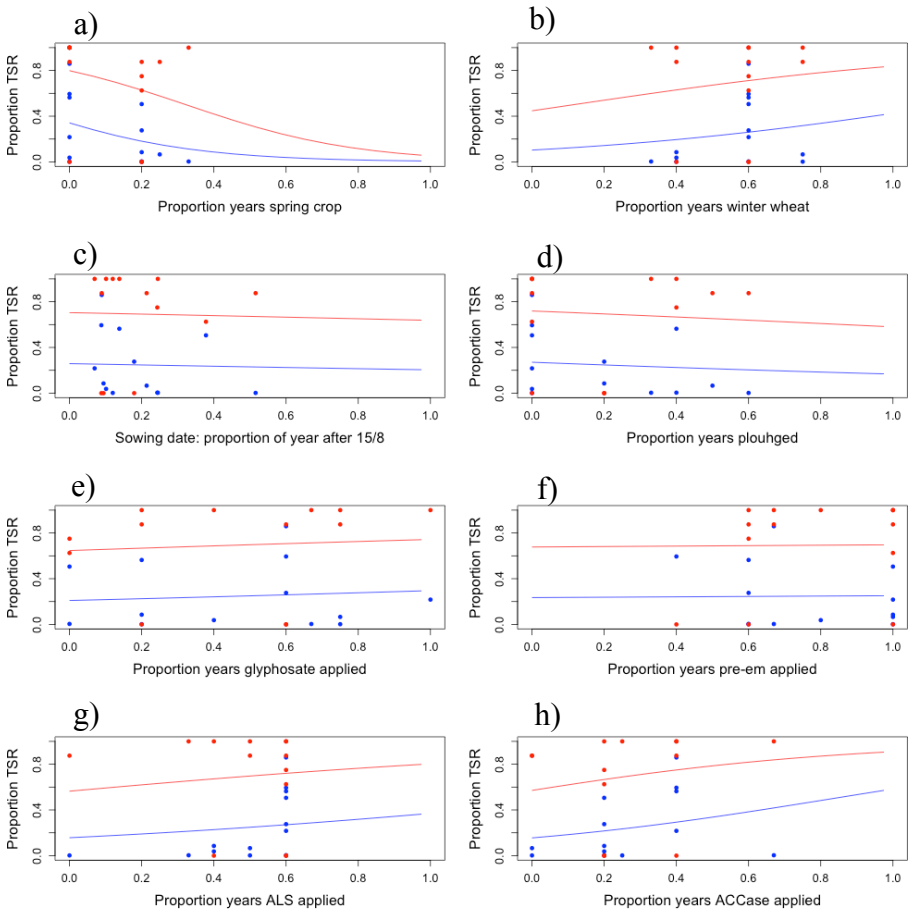


Figure 4.28: Relationships between the proportion of ACCase resistance for model (blue) and field (red) data and the frequency of management applied: (a) proportion of years with a spring crop sown, (b) proportion of years with winter wheat crop sown, (c) winter wheat sowing date as a proportion of the year after 15/8, (d) proportion of years ploughed, (e) proportion of years when an Glyphosate was applied, (f) proportion of years in which a pre-emergence herbicide was applied, (g) proportion of years when an ALS herbicide was applied, and (h) proportion of years when an ACCase herbicide was applied. Graph letters labeled with an asterisk (*) indicates a significant relationship of the resistance measure and the proportion management factor. Solid lines indicate a significant ($P < 0.05$) relationship between the resistance measure and management. Dashed lines coloured indicate a non-significant ($P < 0.2 > 0.05$) relationship between the resistance measure and management.

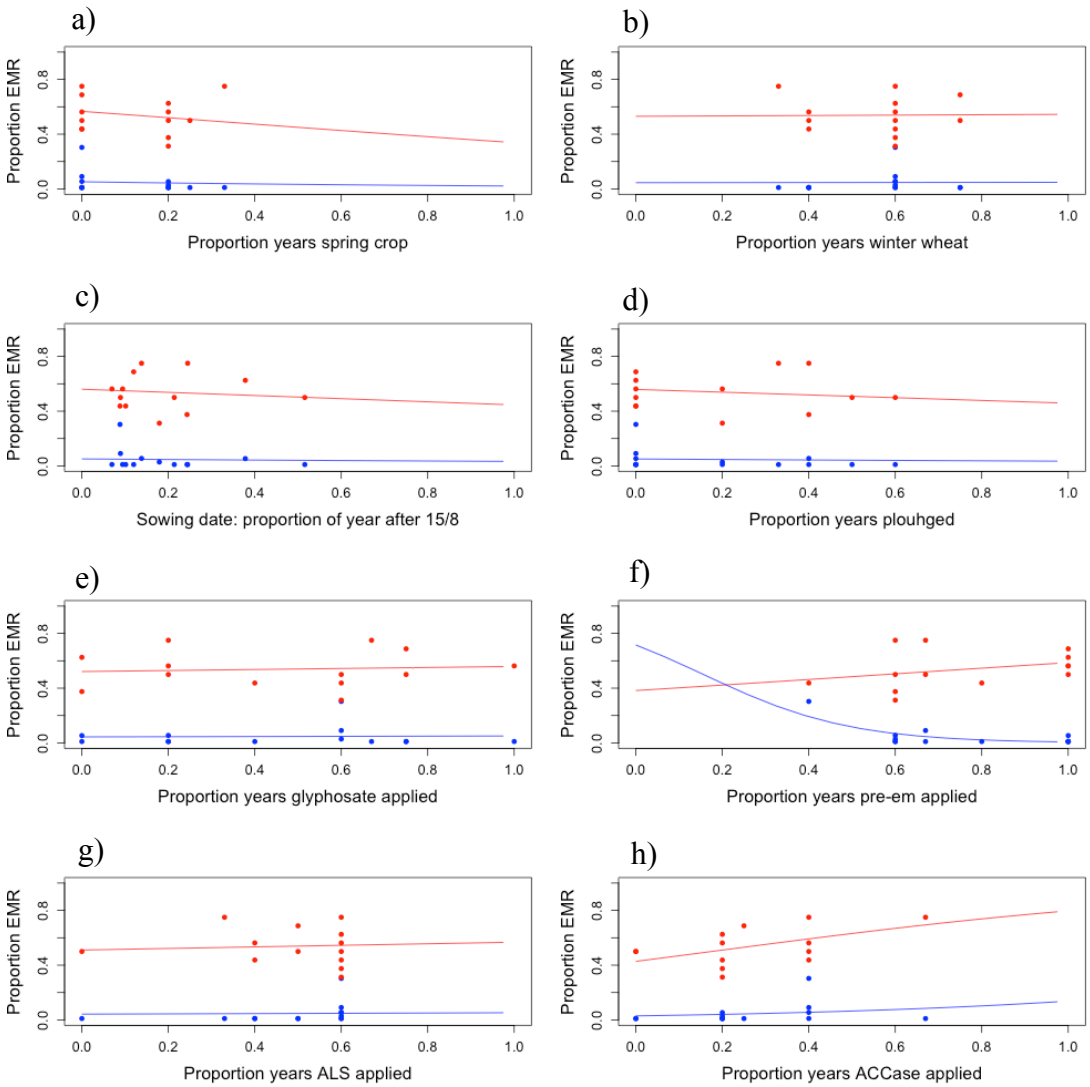


Figure 4.19: Relationships between the proportion of ACCase EMR for model (blue) and field (red) data and the frequency of management applied: (a) proportion of years with a spring crop sown, (b) proportion of years with winter wheat crop sown, (c) winter wheat sowing date as a proportion of the year after 15/8, (d) proportion of years ploughed, (e) proportion of years when an Glyphosate was applied, (f) proportion of years in which a pre-emergence herbicide was applied, (g) proportion of years when an ALS herbicide was applied, and (h) proportion of years when an ACCase herbicide was applied. Graph letters labelled with an asterisk (*) indicates a significant relationship of the resistance measure and the proportion management factor. Solid lines indicate a significant ($P < 0.05$) relationship between the resistance measure and management. Dashed lines coloured indicate a non-significant ($P < 0.2 > 0.05$) relationship between the resistance measure and management.

4.4.6 Prediction of resistance in adjacent years

4.4.6.1 ALS resistance, TSR & EMR in adjacent years

The relationship between ALS TSR predicted from the model and identified from the field data had an intercept of 0.07 and odds ratio ("slope") of 1.49 (Figure 4.20a); this combination of intercept and odds ratio is indicative that the model significantly (Figure 4.20c, (ANOVA, P = 0.009)) under predicts TSR in populations when compared to the field. Similarly ACCase EMR exhibited an intercept of 0.12 and odds ratio of 1.33 (Figure 4.20b), indicating that the model significantly (Figure 4.20d, (ANOVA, P = 0.6)) over predicts EMR regardless of the level of EMR identified in field data.

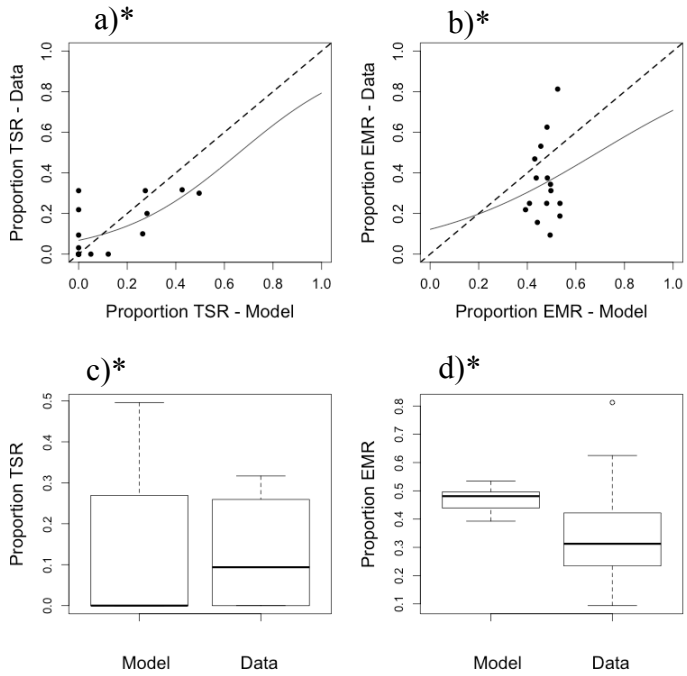


Figure 4.20: Proportion of individuals possessing ALS resistance, TSR and EMR in newly produced seed predicted from modelling compare to the proportions in newly produced seed from field data. (a) ALS TSR model/field data correlation, (b) ALS EMR model/field data correlation, (c) ALS TSR values, and (d) ALS EMR values. If graphs a, or b, or c are marked with an asterisk (*), this indicates that there is a significant correlation between modelling and field data; if graphs c or d are marked with an asterisk (*), this indicates that there is a significant difference between the values produced from modelling and field data. The dashed black line on a and b represents a perfect correlation between field and modelling data.

4.4.6.2 ACCase resistance, TSR & EMR in the year t+1 from year t year

The relationship between ACCase TSR predicted from the model and identified from the field data had an intercept of 0.27 of odds ratio ("slope") of 1.23 (Figure 4.21a); this combination of intercept and odds ratio is indicative that the model significantly (Figure 4.21c, (ANOVA, P = 0.005)) under predicts TSR for field populations in which TSR is at a low level, and over predicts TSR for field populations in which TSR is at a high level. Similarly ACCase EMR exhibited an intercept of 0.01 and odds ratio of 8.07 (Figure 4.21b), indicating that the model significantly (Figure 4.21d, (ANOVA, P = 0.009)) under predicts EMR regardless of the level of EMR identified in field data.

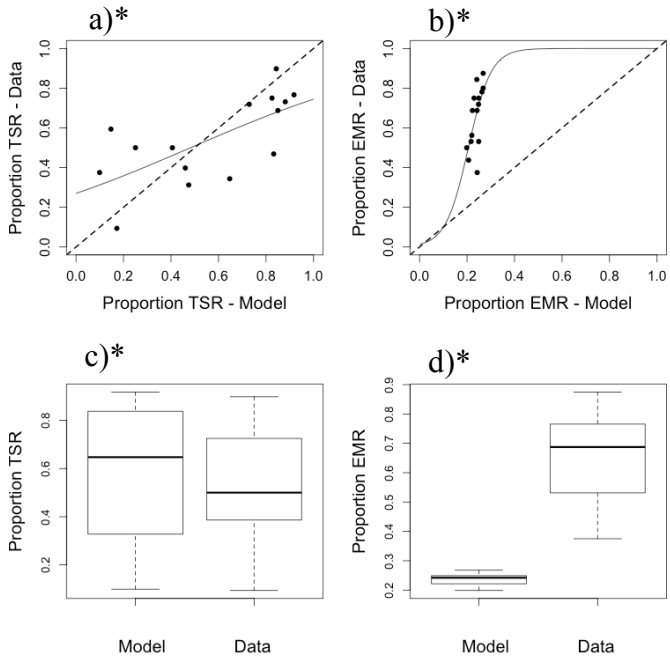


Figure 4.21: Proportion of individuals possessing ACCase TSR and EMR in newly produced seed predicted from modelling compare to the proportions in newly produced seed from field data. (a) ACCase TSR model/field data correlation, (b) ACCase EMR model/field data correlation, (c) ACCase TSR values, and (d) ACCase EMR values. If graphs a, or b, or c are marked with an asterisk (*), this indicates that there is a significant correlation between modelling and field data; if graphs c or d are marked with an asterisk (*), this indicates that there is a significant difference between the values produced from modelling and field data. The dashed black line on a and b represents a perfect correlation between field and modelling data.

4.5 Discussion

4.5.1 Effect of management on ALS and ACCase resistance

Two approaches were used to assess the effect that weed management has on ALS and ACCase resistance index (RI), TSR allele frequency and EMR index; these approaches were the effect that the frequency of management application between 2007 and 2014, and the analysis of data between adjacent years in relation to management.

Of the thirteen UK fields studied that had ALS and ACCase RI, TSR and EMR index information (2012-2014) and weed management history (2007-2014), those that practiced greater frequencies of spring cropping exhibited significantly lower ALS and ACCase RI values, as well as non-significantly lower ALS and ACCase TSR allele frequencies: Increased spring cropping had no effect on ALS and ACCase EMR index. Previously, spring cropping has been estimated to reduce *A. myosuroides* infestations in the field by 78-96% (Lutman *et al* 2013). Coupled with the results of this study, planting increased frequencies of spring crops is not only an effective practice in reducing infestations of *A. myosuroides,* but also an effective means of slowing the evolution of ALS and ACCase resistance. Spring cropping does have limitations, with spring crops being more difficult to establish on heavy soils and there being fewer herbicides available for spring crops (Lutman *et al* 2013). In contrast, when assessing the effect of spring cropping on samples collected in adjacent years (i.e. years t and t+1) fields planting a spring crop only exhibited a significant difference in ALS RI when compared to those planting a winter crop after October 20th. The identification of few significant effects of spring cropping in a single year may be indication that spring cropping may have to be practiced over an

extended period to exhibit an effect upon ALS and ACCase resistance and resistance mechanisms. Alternatively, the lack of significant effect of spring crops may result from that fact that only a small number of populations were studied; more populations should be sampled to confirm this finding.

Of the thirteen UK fields studied that had ALS and ACCase RI, TSR and EMR index information (2012-2014) and weed management history (2007-2014), the increased planting of winter wheat over this period exhibited no significant effect on ALS and ACCase RI, TSR or EMR. However in adjacent years (t and t+1), fields planting winter wheat exhibited a significant increase in ALS RI and TSR allele frequency and a non-significant increase in EMR, while significantly lower levels of ACCase RI and TSR allele frequency were observed with winter wheat planting. The identification of greater frequencies of ALS resistance in fields that had sown winter wheat is unsurprising, as the application of ALS herbicides are applied in winter wheat crops within the UK (Lutman *et al* 2013). Therefore, it would be expected that fields that have more frequently incorporated more winter wheat crops into their management regime will exhibit higher frequencies of ALS resistance, but this is not the case here. Again, the lack of significant effect of winter wheat sowing may result from that fact that only a small number of populations were studied.

Additionally, fields in which the crop was sown later in year t+1 exhibited significantly higher levels of ALS RI, as well as non-significantly higher levels of ALS TSR and EMR, although no effect of later sowing over longer periods (2007-2014) was observed. Fields sowing crops later between 2007 and 2014 did exhibit significantly lower levels of ACCase EMR (but not RI or TSR), and between

adjacent years (i.e. t and t+1) ACCase EMR index significantly increased if the crop was sown after October 20th. This result is surprising, as later sowing of crops is advocated as effective method reducing *A. myosuroides* infestations in the field by a mean of 33% (although the range of efficacy is between -71% and 97%) (Lutman *et al* 2013). However, the observed result may be as a result of how the data has been categorised and analysed. Later sowing dates may equate to higher resistance, as most populations in this category were winter wheat, whereas most of those before October 20th are non-wheat crops (e.g. oilseed rape). Therefore, crops sown after October 20th may exhibit higher levels of ALS resistance, more as a result of the planting of winter wheat crops and application than the sowing date itself. Alternatively, this result may be an indication that delayed sowing may reduce the level of *A. myosuroides* infestation, but not the level of resistance within the population.

Fields that practiced ploughing more frequently more between 2007 and 2014 exhibited lower (non-significant) levels of ALS RI and ACCase EMR index. Between adjacent years (t and t+1), fields that practiced minimum tillage cultivation exhibited non-significantly higher levels of ALS RI, while those that practiced ploughing exhibited non-significantly higher levels ACCase TSR. These findings for the effect of cultivation on the levels of ALS and ACCase resistance and resistance mechanisms are difficult to interpret, as it has been estimated that ploughing can increase *A. myosuroides* infestations by 82% or decrease them by up to 96% (Lutman *et al* 2013). The quality of ploughing - how well the soil is inverted - is important when determining the effect of ploughing on resistance. This information that is not recorded by the farmer so is unattainable; so again, the lack of significant effect of

ploughing may result from that fact that only a small number of populations were studied and that full details regarding ploughing could not be collected.

Increased application of glyphosate between 2007 and 2014 exhibited non-significant increases in ALS RI and EMR index, and between adjacent years (t and t+1) only non-significant higher levels of ALS TSR were observed (no effect of glyphosate application was observed for ACCase RI, TSR or EMR index). It is speculated in this instance, that there is no link between glyphosate and ALS resistance; but that the more problematic the *A. myosuroides* resistance problem in the field, the more glyphosate the farmer will apply. No effect of pre-emergence herbicide application was observed for ALS and ACCase RI, TSR allele frequency or EMR index between 2007 and 2011 or between adjacent years except the application of pre-emergence herbicides exhibiting a non-significant increase in ALS RI when applied. As with ploughing the application of pre-emergence herbicides can be variable, depending upon weather variables, soil factors and also which pre-emergence herbicides were applied. In the analysis conducted here, all pre-emergent herbicide applications were treated as one pre-emergent herbicide group. Dividing the pre-emergent herbicide by MOA for example, may give a clearer indication of how different pre-emergent MOA affect ALS and ACCase resistance evolution.

Increased application of ALS post-emergence herbicide application e between 2007 and 2014 had no effect on the levels of ALS and ACCase RI, TSR allele frequency or EMR index identified, although between years (t and t+1) ALS application leads to a significant increase in RI, TSR and non-significant increase in ALS EMR. Increased ACCase post-emergence herbicides application led to a significant

increase in ACCase RI (2007-2014) and a significant increase in ACCase TSR between years (t and t+1). This finding is not a surprise, as ALS herbicides are applied in almost all winter wheat crops in the UK (Moss 2013). Having said that, no positive correlation was observed between the frequency of ALS herbicide applied and the level of ALS resistance. Increased applications of ACCase herbicide also had no effect on the levels of ACCase resistance (or ALS resistance). This contrasts to the findings of Evans *et al* (2015) studying the frequency of glyphosate resistant *Amaranthus tuberculatus* in 105 fields from Illinois (USA) identified that populations with the greatest frequencies of glyphosate resistant *A. tuberculatus* were associated with frequent applications of the herbicide of interest (glyphosate). Alternatively, significantly lower levels of ACCase RI and non-significant levels of ACCase TSR and EMR between years, and non-significantly lower levels of ALS TSR between 2007 and 2014 and significantly lower levels in ALS TSR is caused when an ALS and ACCase herbicide is applied respectively.

More widely, these results agree with studies of cultural management (Lutman *et al* 2013) and modelling that state that more heterogeneous management strategies will reduce the evolution of resistance (Richter 2002). The results of this study provide important information in how these management practices effect the levels of ALS and ACCase resistance, which can be incorporated into integrated weed management (IWM) schemes to make them effective in controlling ALS and ACCase resistance in *A. myosuroides*. However, as mentioned above, it has to be acknowledged that a limited number of populations have been studied which may have limited the statistical power in some cases.

4.5.2 A. myosuroides resistance model

In an attempt to validate the created *A. myosuroides* resistance model, the ability for it to accurately replicate ALS and ACCase resistance data collected from 2011 to 2014 was assessed. After validating the model using two methods ((1) using historical management data to predict the level of resistance in 2011; (2) whether the model parameterized with resistance and management data from one year could predict resistance in the next), it was found that the model was unable to accurately predict which populations would be most resistant

4.5.2.1 Representation of resistance mechanisms

One area of the model that will have a large impact on the output of model is the way in which the mechanisms of resistance are represented within the model: Namely enhanced metabolism. In its current format a single gene that endows some level of resistance to both ALS and ACCase modes of action represents enhanced metabolism. With details of the genetics of enhanced metabolism (a polygenic trait) still largely unknown, it was therefore not unreasonable to treat enhanced metabolism as a single gene trait for both modes of action. One indication from the model not being validated by the field data however is that ALS and ACCase enhanced metabolism are separate mechanisms.

To improve the model, incorporating two enhanced metabolism genes, one for each mode of action, will be a good place to start, eliminating the need to represent enhanced metabolism data from the field for ALS and ACCase as a composite figure. The modelling of enhanced metabolism has been attempted in a number of studies (Gardner *et al* 1998; Renton *et al* 2011). Gardner *et al* (1998) represented metabolic

resistance using a quantitative genetics framework (e.g. selection from a normal distribution), while Renton *et al* (2011) modelled enhanced metabolism as a polygenic trait. Either approach could be incorporated into the model presented here in an attempt to improve the accuracy of the model. What would be more beneficial would be to use the model to validate the results of recent publications that have identified certain aspects about enhanced metabolism i.e. the estimated that the minimum number of genes involved in ALS and ACCase enhanced metabolism (Rosenhauer *et al* 2015) and identification of the regulatory gene *AmGSTF1* (Cummins *et al* 2013).

4.5.2.2 Parameter estimates and validatory data

Although a number of herbicide resistance models have been published for a number of species (Richter *et al* 2002; Diggle *et al* 2003; Neve *et al* 2003a, 2003b; Neve 2008; Jacquemin *et al* 2009; Neve *et al* 2010; Richter *et al* 2012; Bagavathiannan *et al* 2014), this is one of the first to attempt (and the first for *A. myosuroides*) to validate the model using field data. Another factor that will have impacted the modelling output is the parameter estimates used within the model (estimated from published literature) and the quality of the field data used to validate it. To fully understand the affect of these on the model, a greater range of each parameter estimates would have to be used within the model, and the model would have to be compared to a greater number of field data studies.

4.6 Conclusion

The seventeen UK populations studied between 2012 and 2014 exhibit a wide range of changes in phenotypic, target-site and enhanced metabolic resistance (chapter 3).

To fully understand the trends in phenotypic, target-site and enhanced metabolic resistance described, they need to be evaluated in light of the weed management that has been applied. Assessing the changes in ALS and ACCase resistance and resistance mechanism between 2012 and 2014 shows that ALS resistance is increasing as a result of target-site resistance. ACCase is not increasing. Increases in ALS resistance due to the mechanism of target-site resistance is associated with the frequent planting winter wheat crops, whereas the planting of non-winter wheat crops is associated with increased ACCase resistance due to both target-site resistance and enhanced metabolism. Increased planting of spring crops significantly reduces the level of ALS phenotypic resistance, and non-significantly decreases the levels of ACCase phenotypic resistance. Through the findings of this study, and more long-term epidemiological studies such as this, a greater understanding the temporal evolution of *A. myosuroides* herbicide resistance in relation to management can be distinguished, so that more effective *A. myosuroides* resistance control strategies can be developed.

5.0 Investigating the segregation of Pro-197-Thr target-site mutations in the acetolactate synthase (ALS) gene of *Alopecurus myosuroides.*

5.1 Introduction

5.1.1 Acetolactate synthase (ALS) target-site resistance

Acetolactate synthase (ALS) is the first anabolic enzyme in the biosynthesis of the branch chained amino acids leucine, isoleucine and valine (Tranel and Wright 2002). ALS inhibiting herbicides, of which there are five classes - sulfonyureas, imidazolinones, sulfonylamino-carbonyl-triazolinones, pyrimidinyl(thio)benzoates, and triazolopyrimidines (HGCA 2010) - induce plant death by preventing the synthesis of ALS end-products (leucine, isoleucine, and valine), disrupting protein synthesis, and causing 2-ketobutyrate to accumulate (Yu and Powles 2014).

ALS target-site resistance (TSR) results from point mutations in the ALS gene. These point mutations cause amino acid substitutions that change enzyme structure to prevent normal enzyme-herbicide binding (Tranel and Wright 2002; Powles and Yu 2010). ALS TSR has been characterized in 50 of the 139 weed species with known ALS resistance (Heap *et al* 2015). To date, a total of twenty-four different ALS amino acid substitutions conferring resistance have been identified at eight amino acid positions: Ala-122 (3 different amino acid substitutions), Pro-197 (11), Ala-205 (1), Asp-376 (1), Arg-377 (1), Trp-574 (3), Ser-635 (3), and Gly-654 (1) (Heap *et al* 2015). Of these nucleotide positions, polymorphisms at position Pro-197 are most frequent in occurrence (Yu and Powles 2014), with eleven different amino

acids substitutions being established across thirty-two species (Table 5.1) (Heap *et al* 2015).

Table 5.1: List of weed species possessing a Pro-197 target-site mutations and the amino acid substitutions identified for each. (Source: Tranel, P.J., Wright, T.R, and Heap, I.M. Mutations in herbicide-resistant weeds to ALS inhibitors. Online http://www.weedscience.com. 3/9/2015).

Species	Amino acid(s) substituted for Pro-197
Alopecurus myosuroides	Thr
Amaranthus blitoides	Ser
Amaranthus retroflexus	Leu
Anthemis cotula	Gln, Thr, Ser, Leu
Apera spica-venti	Ala, Asn, Ser, Thr
Brassica tournefortii	Ala Ser
Bromus tectorum	Ser, Thr
Capsella bursa-pastoris	His, Thr, Ser, Leu
Chrysanthemum coronarium	Ser
Conyza canadensis	Ala
Descurainia sophia	Ala, Thr, Ser, Leu, His, Tyr
Helianthus annuus	Leu
Hordeum murinum ssp. leporinum	Ser, Thr
Kochia scoparia	Ala, Arg, Gln, Leu, Ser, Thr
Lactuca serriola	His, Thr
Lindernia dubia (=Lindernia dubia var. major)	Ala, Ser
Lindernia micrantha	Gln, Ser
Lindernia procumbens	Gln, Ser
Lolium rigidum	Ala, Arg, Gln, Leu, Ser
Monochoria vaginalis	Ser
Myosoton aquaticum	Glu, Ser
Papaver rhoeas	Ala, Arg, His, Leu, Ser, Thr
Raphanus raphanistrum	Ala, His, Ser, Thr
Sagittaria trifolia	Ser
Salsola tragus	Gln
Schoenoplectus juncoides	His, Ser, leu
Schoenoplectus mucronatus (=Scirpus mucronatus)	His
Sinapis arvensis	Ser
Sisymbrium orientale	Ile
Sonchus asper	Leu
Stellaria media	Gln
Thlaspi arvense	Leu

5.1.2 Pro-197-Thr target-site mutations in Alopecurus myosuroides

In *A. myosuroides,* and eleven other weed species (Table 5.1)*,* target-site resistance to ALS modes of action (MOA) can be bestowed by a Proline to Threonine amino acid substitution at amino acid position 197 of the ALS protein (Pro-197-Thr). This amino acid change results from a substitution in one of the nucleotides that code for proline at position 197 of the ALS enzyme: In *A. myosuroides,* the nucleotide substituted is cytosine for adenine (i.e. ACC for CCC). Only two target-site mutations endow ALS resistance in *A. myosuroides*, Pro-197 and Trp-574. The preferential selection of these mutations may be a reflection of herbicide use. Sulfonylurea herbicides are widely used to control *A. myosuroides* - mesosulfuron-methyl + iodosulfuron-methyl-sodium (commercial name Atlantis WG) was the most widely used herbicide formulation on wheat crops in the UK in 2012 (DEFRA 2013) - and sulfonylurea herbicides are known to preferentially select Pro-197 mutations, while sulfonylurea + imidazolinones select for Trp-574 (Yu and Powles 2014).

The prevalence of Pro-197-Thr ALS target-site mutations in *A. myosuroides* at any scale is not well established. In Germany, Hess *et al* (2012) found that Pro-197-Thr mutations were present in 0%, 31%, and 20% of 32, 51, and 21 populations sampled from three regions, respectively. In the UK, Moss *et al* (2014) established that 7% of 570 plants from 19 random populations sampled between 2009 and 2011 possessed Pro-197-Thr target-site mutations. Furthermore, research presented in this thesis has identified that of the ninety-two *A. myosuroides* populations studied from across England, thirty-three (36%) possessed Pro-197-Thr target-site mutations; Pro-197-Thr target-site mutations were identified in 19% of the 736 plants studied (chapter 2).

5.1.3 Mendelian inheritance and Hardy-Weinberg equilibrium

Three laws of Mendelian inheritance determine how genetic variation, alleles, at each locus of a gene are inherited by the next generation: 1) offspring inherits one allele from each parent; 2) dominant alleles determine phenotype; 3) alleles from each parent will end up in different gametes in expected proportions (Campbell and Reece 2005). For example, when two individuals heterozygous (recessive (p): dominant (q)) for a trait (pq) reproduce, they will bear progeny with a genotype 25% homozygous recessive (pp), 50% heterozygous (pq), and 25% homozygous dominant (qq). Phenotypically, the dominant trait is expressed in 75% of individuals, due to the dominant allele being present in heterozygous and homozygous dominant individuals.

G. H. Hardy and Wilhelm Weinberg first described the principle of Hardy-Weinberg equilibrium in separate publications in 1908. It states that in the absence of external factors, genetic variation within a population will remain constant (Campbell and Reece 2005). In order for Hardy-Weinberg equilibrium to be achieved within a population, five conditions must be met: the population must be large, there must be no gene flow between different populations, the allele in question must not be mutating within the population, the population must be randomly mating, and the population must not be under natural selection (Campbell and Reece 2005). If these conditions are met, the frequency of three possible genotypes within a population, homozygous allele p (p^2), heterozygous allele p and heterozygous allele q (2pq), and homozygous allele q (q^2) can be described by the Hardy-Weinberg equilibrium equation (Equation 5.1).

$$p^2*(2pq)*q^2 = 1$$

(Equation 5.1)

5.1.4 Studies investigating the zygosity of target-site mutations

Alleles that confer resistance allow a plant to survive a treatment of herbicide, though this ability to survive may come at a cost. When a resistant individual is not in the presence of herbicide, a 'cost of resistance' (a limit on fitness (i.e. fecundity) and selection) may be observed (Vila-Aiub *et al* 2011). Target-site mutations that confer resistance may also compromise target-enzyme functionality, leading to a cost of resistance: Homozygous lethality can be thought of as a total loss of fitness.

The lethality of homozygous Pro-197 mutations has not been directly studied for any weed species. However, no lethal affect has been observed for homozygous Pro-197-Ser mutations in populations of *Raphanus raphanistrum* (Li *et al* 2012; Yu *et al* 2012) and *Arabidopsis thaliana* (Roux *et al* 2004), and no lethal affect has been observed for homozygous Pro-197-Thr mutations in populations of *Lolium rigidum* (Collavo and Sattin 2014), *Papaver rhoeas* (Délye *et al* 2011), and *R. raphanistrum* (Yu *et al* 2012). In one study of 136 *A. myosuroides* plants from six UK populations, Pro-197-Thr mutations were identified in 41 plants from two populations (one of which was a standard Pro-197-Thr resistant population). 23 of the 41 Pro-197-Thr mutations were homozygous (Marshall *et al* 2013). In contrast to this finding, during the characterization of ALS resistant *A. myosuroides* in the UK, Marshall and Moss (2008) noted that all individuals (42 mesosulfuron-methyl-sodium + iodosulfuron-methyl resistant individuals from seven populations) appeared heterozygous for the Pro-197-Thr mutation.

From the UK survey of *Alopecurus myosuroides* conducted in 2011 (Chapter 2) it was noted that there were no homozygous Pro-197-Thr present in the 736 plants tested across ninety-two populations. This lead to the question whether *A. myosuroides* homozygous Pro-197-Thr mutations are lethal, or at least, not in Hardy-Weinberg equilibrium?

5.2 Objectives

Work reported in this chapter investigates the potential lethality of homozygous Pro-197-Thr target-site mutations within the ALS gene of *Alopecurus myosuroides.* For each of two UK populations, heterozygous Pro-197-Thr *A. myosuroides* plants were identified and crossed in pairs to produce progeny with an expected Mendelian inherited Pro-197-Thr genotype of 25% wildtype, 50% heterozygous, and 25% homozygous individuals.

To confirm the presence of these proportions within the progeny, wildtype, heterozygous, and homozygous individuals were identified through phenotyping and genotyping of plants at the two-leaf stage. To determine whether homozygous seeds are produced (but are not viable and do not germinate) or die shortly after germination (so no homozygous plants are phenotyped), the ratio of wildtype, heterozygous, and homozygous individuals present at germination was also determined via the genotyping of seedlings from a germination test. To further investigate the effect of lethal homozygous Pro-197-Thr target-site mutations, simulation models were used to compare how Pro-197-Thr mutations evolve when homozygous mutations are lethal vs. non-lethal.

5.3 Materials and Methods

5.3.1 Plant material

In July 2012, as part of annual ALS and ACCase resistance monitoring (chapter 3), seed from fifteen UK *A. myosuroides* populations were sampled (see section 3.3 for sampling details). Of the sampled populations two, GBR_12075 and GBR_12105, that had previously been identified from the survey in 2011 (chapter 2) as possessing heterozygous Pro-197-Thr mutations and low levels of ALS enhanced metabolism were selected for use, as they were also expected to possess Pro-197-Thr mutations in 2012 (Table 5.2).

Table 5.2: 2011 survey information used to select populations used for the experiment. For both populations, the location of the sampled field, ALS resistance phenotype, number of wildtype (wild), heterozygous (het), and homozygous (hom) Pro-197-Thr and Trp-574-Leu ALS mutations (out of 8 plants), and mean percentage (out of 8 plants) of mesosulfuron metabolised by the population (i.e. level of ALS enhanced metabolism) is indicated. (Values are taken from raw data generated in Chapter 2).

Population	Sampling Location	ALS Phenotype	ALS Target-Site Resistance						Mean % of mesosulfuron metabolised
			Pro-197-Thr			Trp-574-Leu			
			Wild	Het	Hom	Wild	Het	Hom	
GBR_11075	Hardwick Northamptonshire NN9 5AW	Resistant	5	3	0	8	0	0	13
GBR_11105	Edgware Greater London HA8 8QS	Resistant	4	4	0	8	0	0	5

5.3.2 Identification of phenotypically resistant plants for pairwise crossing

In October 2012, two seeds were sown into each well of a 308 well modular tray. One tray was established for each population (GBR_12075 and GBR_12105). Wells were filled with a 2:1:1 mix of J. Arthur Bower's topsoil (English loam blended with organic matter and nutrients, pH: 6.5 – 7.5), 0.5 Levington growing media: M2 (pH: 5.5 – 6, N: 200, P: 150, K: 200 mg/liter), and 0.25 J. Arthur Bower's silver sand (lime-free washed silica sand). Both trays were maintained on a bench within a

glasshouse compartment (22°C day/16°C night; 14 hour photoperiod) and watered regularly; fertilizer was applied when required. Three weeks after sowing, germinated seedlings were thinned to one plant per well and plants were treated with a single dose – the recommended UK field rate - of mesosulfuron-methyl (14g a.i ha^{-1}) + iodosulfuron-methyl-sodium (2.4g a.i ha^{-1}) and the adjuvant Biopower (0.27 kg a.i ha^{-1}). Herbicide was applied using a Berthoud 2000 knapsack sprayer. The sprayer was set to apply herbicide at a pressure of 300 kPa while walking at a fixed pace of 3 kph. A spray volume of 200L/ha was delivered through a Hypro standard flat fan tip (110 degrees F110-03 ultra-blue nozzle), positioned 40cm above the height of the modular tray. Plants were returned to glasshouse compartment and watered regularly after herbicide application. Three weeks after herbicide application, the number of surviving plants was recorded (Table 5.3). Of the surviving resistant plants, forty-eight plants per population were randomly selected and a 2cm section of leaf tissue was taken from each for subsequent Pro-197 ALS TSR genotyping (method described in section 2.3.3).

Table 5.3: Number of resistant and susceptible individuals identified within populations GBR_12075 and GBR_12105 when treated with a mesosulfuron-methyl (14g a.i ha^{-1}) + iodosulfuron-methyl-sodium (2.4g a.i ha^{-1}).

Population	Total Plants	Number Resistant	Number Susceptible
GBR_12075	146	116	30
GBR_12105	210	94	116

5.3.3 Crossing plants for seed production

Of the 48 plants genotyped from each population (GBR_12075 and GBR_12105) that survived treatment with mesosulfuron-methyl (14g a.i ha^{-1}) + iodosulfuron-methyl-sodium (2.4g a.i ha^{-1}), 37/48 and 40/48 plants, respectively possessed heterozygous Pro-197-Thr mutations, making them suitable for performing pairwise

crosses to produce seed with an expected 1:2:1 genotypic frequency of wildtype: heterozygous: homozygous individuals (Table 5.4).

Table 5.4: ALS target-site genotypes for 48 ALS resistant plants from populations GBR_12075 and GBR_12105 Wildtype survival indicates enhanced metabolism as the mechanism of resistance.

Population	Wildtype	Pro-197-Thr		Trp-574-Leu		Pro-197-Thr + Trp-574-Leu		No Result
		Het	Hom	Het	Hom	Het	Hom	
GBR_12075	6	37	0	1	0	1	0	2
GBR_12105	2	40	0	4	0	0	0	2

For each population, twenty-four plants with heterozygous Pro-197-Thr mutations were randomly chosen and paired to create twelve independent crosses. Each pair of plants was transplanted into a 6" pot filled with the potting mix described in section 5.3.2. To prevent pollen exchange between crosses, a pollen proof bag (held in place by an elastic band and supported by two wooden canes (Figure 5.1)) was placed over each pot. Each pot was stood in a saucer so they could be watered without disturbing the plants. When required, a liquid nutrient feed was also supplied via the saucer. Between April and July 2013, pots were randomly placed 1m apart within a polythene tunnel to produce seed. When mature, seed was collected from each plant within a cross into separate paper bags and stored in a drying room (relative humidity = 15%) until use.

5.3.4 Phenotyping of seed produced from crosses

For both populations the amount of seed produced by each cross was low, with estimated seed quantities ranging from 250 – 3075 seeds per cross. Seed from three of each of the GBR_12075 and three GBR_12105 crosses (those with good levels of seed production) were phenotyped to identify the frequency of mesosulfuron-methyl

+ iodosulfuron-methyl-sodium resistance. As seed from each of the plants within a paired cross was collected separately (section 5.3.3), seed produced from each plant in a paired cross was phenotyped separately.

Figure 5.1: Two *A. myosuroides* plants heterozygous for the Pro 197 ALS mutation grown in a single pot with a pollen proof cover.

For each of the three crosses per population, four hundred seeds, 200 from each plant in a cross (plus susceptible and resistant control populations), were germinated in petri dishes (100 seeds per petri dish). Into each petri dish was placed two Whatmann 8.5mm number 1 filter paper to which was added 3.5ml of a 2g/L solution of potassium nitrate (KNO^{-3}). Petri dishes were then incubated at 23°C, day 9°C night (12:12hours) for two weeks.

For each of the three crosses per population (12 in total plus the susceptible and resistant control populations), 5 replicate FP9 pots - filled with the potting mix

described in section 5.3.2 - were sown with 8 germinated seedlings, giving seventy pots in total. Pots were arranged in a completely randomised block design in a glasshouse compartment (temperature: $22^{o}c/16^{o}c$; photoperiod: 14 hours) and watered regularly. Before herbicide application a 2cm piece of leaf was sampled from each plant for subsequent Pro-197 TSR genotyping (method described in section 5.3.5). Two weeks after sowing, all plants were treated with mesosulfuron-methyl (14g a.i ha^{-1}) + iodosulfuron-methyl-sodium (2.4g a.i ha^{-1}) and the adjuvant Biopower (0.27g a.i ha^{-1}) (method detailed in section 5.3.2). Three weeks after herbicide application the number of surviving plants was recorded.

5.3.5 Pro-197-Thr Target-site analysis

5.3.5.1 DNA extraction

Each piece of leaf tissue was placed into a separate well of a 96-deep-well plate. To each well a stainless steel bead and 400µL of extraction buffer (100mM Tris(HCl) and 1 M KCl, pH 9.5) were added. Plates were shaken (30rpm for 10 minutes) using a Qiagen TissueLyser II, and centrifuged (4000rpm for 10 minutes) using a Sigma laboratory 4K15C centrifuge to extract the DNA. 100µl of supernatant was transferred into a new 96-well plate, from which a second plate containing 5µl supernatant and 250µl of Sigma-Aldrich W3500 sterile filtered tissue culture water was produced. The 100µl plate was stored at -80°C, while the 5µl plate was used as the PCR DNA template.

5.3.5.2 PCR Conditions for identifying Pro-197 TSR mutations

To identify single nucleotide polymorphisms (SNP) at position Pro-197 of the ALS gene, a 140bp biotinylated fragment was amplified using forward and reverse

primers (forward: 5'-GTGCTACCAACCTCGTCTC-3'; reverse: 5'-GGAGCGGGTGACCTCTACAAT-3'). A Master mix consisting of 10µl forward primer, 10µl reverse primer, 1030µl Sigma-Aldrich W3500 sterile filtered tissue culture water and 2500µl of taq polymerase was produced. 5µl of DNA template and 20µl master mix was combined in a PCR plate (total volume = 25µl) using a Beckman Coulter Biomek 3000 workstation. The plate was sealed and centrifuged using an Eppendorf 5810R centrifuge at 4000rpm for 1 minute. Amplification of the target region was carried out using an Eppendorf Mastercycler Gradient PCR machine set to denature at 94°C for 30s for 45 cycles, anneal at 60°C for 30s for 45 cycles, and elongate at 72°C for 40s for 45 cycles. PCR products were stored at 4°C until use.

5.3.5.3 Pyrosequencing

The PCR product was split into two 12µl aliquots for cleaning and sequencing. 70µl of binding buffer and beads was added to each aliquot and the mixture was shaken for five minutes. 12µl of annealing buffer containing the Pro-197 sequencing primer (5'-ATGGTCGCTATCACGGGACAGGTT-3') were added to each well of a Pyrosequencing plate. The bead bound DNA was washed using a Qiagen Pyromark Q96 workstation in 70% ethanol, sodium hydroxide (NaOH), and wash buffer before being transferred to the sequencing plate and dried at 80°C for two minutes. The Qiagen Pyromark Q96 MD pyrosequencer was set up so that the 10 nucleotides surrounding the single nucleotide polymorphism (SNP) mutation were sequenced. Peaks on the resulting pyrograms indicated the frequency of each nucleotide present.

5.3.6 Germination test

To determine whether homozygous seed are produced (but are not viable and do not germinate) or die shortly after germination (so no homozygous plants are phenotyped), the ratio of wildtype, heterozygous, and homozygous individuals present at germination was also determined via the genotyping of seed and seedlings from a germination test. 2800mL of 0.75% (w/v) agar media was made with addition of 11.2g of potassium nitrate to promote seed germination. Following heating, 100ml of molten agar was poured into each of twenty-eight germination plates (Figure 5.2). After cooling, a lid was placed on each plate and plates were stored in a fridge at 12°C until use. To each of four replicate germination plates, approximately 50 seeds of one of the seven populations (GBR_12075 crosses 6, 10,12; GBR_12105 crosses 2, 9, 10; and a susceptible control) were evenly spread across the agar (seed from each of the plants within a paired cross was collected separately and phenotyped separately (sections 5.3.3 and 5.3.4), however when conducting the germination tests, seed from each of the plants within a paired cross was bulked together due to limited seed). The germination plates were then placed in a glasshouse compartment in a randomised block design (temperature: 22^{o}c/16^{o}c; photoperiod: 14 hours with supplementary lighting). Fourteen days after placing in the compartment, the number of germinated seedlings was recorded. Leaf samples from all germinated seeds were taken to confirm the mutations present by genotyping. (All leaf samples were genotyped for Pro-197 TSR by the methods described in section 3.3.3).

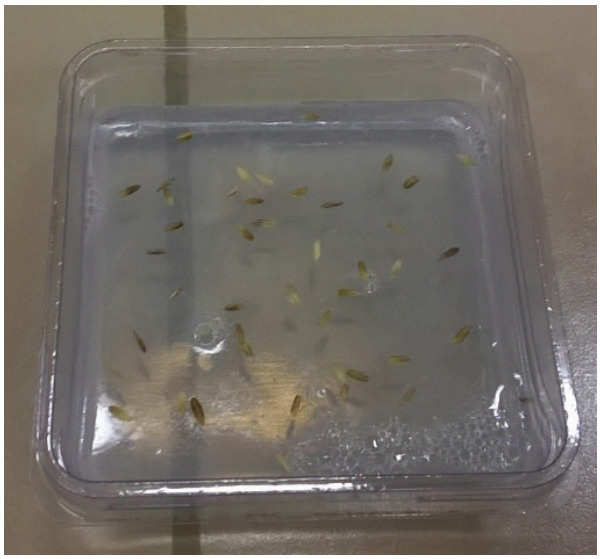


Figure 5.2: Example of the agar germination plate used in the germination test.

5.3.7 Modelling the affect of lethal homozygous Pro-197-Thr mutations

The *A. myosuroides* herbicide resistance model created (Chapter 4, section 4.3.3) was adapted and parameterized to predict the affect that a lethal homozygous Pro-197-Thr ALS mutation has on the evolution of ALS herbicide resistance in *A. myosuroides*. The model was parameterized and implemented as described in section 4.3.3, with the exception of the modifications described below. Within the model, ALS target-site resistance is represented by one diallic, Mendelian inherited gene. In the scenarios tested here, this gene is representative of a Pro-197 allele. To predict the affect that a lethal homozygous Pro-197-Thr ALS mutation has on the evolution of ALS herbicide resistance in *A. myosuroides,* two competing models were created:

1. A model in which homozygous Pro-197-Thr mutations do not exhibit lethality
2. A model in which homozygous Pro-197-Thr mutations are lethal

Each model was run over twenty generations; with the weed management parameters (sowing of continuous winter wheat and application of an ALS herbicide in all years), and initial allele frequencies of ALS TSR, ACCase TSR, and EMR ($1*10^{-6}$) being identical in both. In the original model (section 4.3.2), the proportion of individuals surviving herbicide application is higher for ALS TSR (heterozygous survival = 0.95, homozygous survival = 0.975) than for ALS EMR (heterozygous survival = 0.2, homozygous survival = 0.4). As a result, ALS TSR is selected more rapidly than EMR, and therefore renders effect of ALS EMR negligible in the scenarios tested here.

To account for the lethality of homozygous Pro-197-Thr mutations in model 2, when the seed produced by reproduction for each genotype was calculated (see section 4.4.2 for details), those that possessed a homozygous Pro-197-Thr mutation were multiplied by 0 to simulate lethality. To make the model comparable to previous experimental work conducted using seed samples of known genotypes produced from controlled crosses, the model output used to investigate the affect that lethal homozygous Pro-197-Thr mutations have on the evolution of ALS herbicide resistance in *A. myosuroides* was the seed produced at reproduction after each generation.

5.3.8 Statistical analysis

The phenotyping of the population crosses were analysed using a Fisher's exact test in R (R Development Core Team 2012), comparing the resistant to surviving plants to two ratios; the proportion of wildtype, heterozygous, and homozygous Pro-197-Thr mutations expected if the population is in Hardy-Weinberg equilibrium (3:1),

and the proportion of wildtype, heterozygous, and homozygous Pro-197-Thr mutations expected if homozygous plants are lethal (2:1). The genotype of leaf samples taken from the phenotypic assessment of population crosses was analysed using a Fisher's exact test in R (R Development Core Team 2012), comparing the wildtype: heterozygous: homozygous individuals to two ratios; the proportion of wildtype, heterozygous, and homozygous Pro-197-Thr mutations expected if the population is in Hardy-Weinberg equilibrium (1:2:1), and the proportion of wildtype, heterozygous, and homozygous Pro-197-Thr mutations expected if homozygous plants are lethal (2:1). The genotype of germinated seed from the germination test was analysed using the same method as the genotyping of leaf samples taken from the phenotypic assessment of population crosses.

5.4 Results

5.4.1 Population phenotyping

A Fishers exact test was used to compare the results of the phenotyping (Table 5.5) to the resistant/susceptible ratios in each hypothesis listed in section 5.3.8. Two crosses - one from each population – exhibited no significant difference from either of the 3:1 or 2:1 resistant/susceptible phenotypic ratios. In the remaining four crosses (and for the average across all six crosses) survival was significantly higher than the anticipated 3:1 or 2:1 resistant/susceptible ratios. Even though one caveat for choosing the populations was their absence of non-target-site resistance (NTSR) mechanisms, the significantly higher than expected survival observed may be as a result of the presence of enhanced metabolism.

Table 5.5: The proportion of phenotypically resistant and susceptible individuals to a dose of mesosulfuron-methyl (14g a.i ha^{-1}) + iodosulfuron-methyl-sodium (2.4g a.i ha^{-1}). Significant differences (Fisher's exact test, $p < 0.05$) in the proportion of resistant plants compared to the expected ratios (3:1 ratio if homozygous are non-lethal and 2:1 ratio if homozygous) are indicated by an asterisk (*).

Population	Cross	Total Plants	Phenotype		Proportion Resistant	Comparison to 3:1	Comparison to 2:1
			Susceptible	Resistant			
GBR_12075	6	82	26	56	0.38	0.68	0.87
	10	95	14	81	0.10	0.85	0.00*
	12	100	5	95	0.00*	0.95	0.00*
GBR_12105	2	120	7	113	0.00*	0.94	0.00*
	9	30	7	23	0.81	0.77	1.00
	10	72	0	72	0.00*	1.00	0.00*
Total		499	59	440	0.88	0.00*	0.00*

5.4.2 Genotyping of phenotyped leaf samples

Before phenotyping with mesosulfuron-methyl (14g a.i ha^{-1}) + iodosulfuron-methyl-sodium (2.4g a.i ha^{-1}) a leaf sample was taken from each plant. For each cross/population a number of leaf samples phenotypically resistant and susceptible plants (Table 5.6) were genotyped to test whether any of the resistance was as a result of homozygous Pro-197-Thr mutations. If homozygous individuals are present, they should be in a 1:2 ratio compared to the heterozygous individuals: If homozygous Pro-197-Thr mutations are lethal, all of the resistance should be endowed by heterozygous Pro-197-Thr mutations (Table 5.6).

Table 5.6: Number of homozygous and heterozygous mutations identified in the crosses populations. The total number of leaf samples genotyped is represented (Total). All individuals from in the susceptible column were phenotypically susceptible. All individuals in the EMR, heterozygous and homozygous columns were phenotypically resistant. Enhanced metabolism is anticipated in the EMR individuals as no target-site mutations were identified.

Population	Cross	Total	Susceptible	EMR	Heterozygous	Homozygous
GBR_12075	6	53	8	16	29	0
	10	54	8	24	22	0
	12	50	5	3	42	0
GBR_12105	2	52	7	10	35	0
	9	29	6	11	12	0
	10	45	0	13	32	0
Total		283	34	77	172	0

No homozygous Pro-197-Thr mutations were identified in any of the crosses for either population - adding to the evidence that homozygous Pro-197-Thr mutations may carry lethality. However, not all of the phenotypically resistant plants had resistance endowed by heterozygous Pro-197-Thr mutations. A total of seventy-seven individuals had resistance conferred by enhanced metabolism (as speculated in section 5.4.1).

To observe the true resistant/susceptible phenotypic ratios conferred by Pro-197-Thr mutations, the phenotypically resistant individuals with enhanced metabolism were treated as susceptible and the resistant/susceptible phenotypic ratios were reassessed (Table 5.7).

Table 5.7: The proportion of genotypically resistant individuals with Pro-197-Thr target-site mutations only. Resistant plants are determined as those which are resistant due to heterozygous (RS) or homozygous (RR) Pro-197-Thr target-site mutations. Susceptible individuals are those that died when treated with a dose of mesosulfuron-methyl (14g a.i ha^{-1}) + iodosulfuron-methyl-sodium (2.4g a.i ha^{-1}), or survived herbicide treatment through a mechanism other than a Pro-197-Thr mutation. Significant differences (Fisher's exact test, $P < 0.05$) in the proportion of resistant plants compared to the expected ratios (1:2:1 ratio if homozygous are non-lethal and 2:1 ratio if homozygous are lethal) are indicated by an asterisk (*).

Population	Cross	Total Plants	Susceptible (No TSR)	RS Resistant	RR Resistant	Proportion Resistant	Comparison to 1:2:1	Comparison to 1:2
GBR_12075	6	53	24	29	0	0.55	0.00*	0.03*
	10	54	32	22	0	0.41	0.00*	0.55
	12	50	8	42	0	0.84	0.00*	0.06
GBR_12105	2	52	17	35	0	0.67	0.00*	1.00
	9	29	17	12	0	0.41	0.00*	0.11
	10	45	13	32	0	0.71	0.00*	0.82
Total		283	111	172	0	0.61	0.00*	0.16

As a result of a complete absence of homozygous Pro-197-Thr mutations, five of the crosses exhibited a wildtype to heterozygous genotypic ratio not significantly different ($P > 0.05$) from the ratio 1:2 expected if homozygous Pro-197-Thr mutations are lethal. All crosses exhibited significantly different ($P < 0.05$) genotypic

ratios to the 1:2:1 ratio expected if in Hardy-Weinberg equilibrium. When interpreting the genotype frequencies in relation to phenotype (with metabolically resistant individuals eliminated from the resistant proportion of the phenotype), all but one cross - GBR_12075 cross 6 - exhibited a resistant: susceptible ratio not significantly different ($P > 0.05$) to the 2:1 ratio expected if homozygous Pro-197-Thr mutations are lethal.

5.4.3 Germination test

A germination test was conducted for each of the three crosses from both populations. Germination was low in all crosses. The proportion germinated in each of the six crosses was significantly lower ($P < 0.05$) than the 53% germination of the control population, with the highest level of germination observed in the six crosses was 12% of seeds for GBR_12075 cross 6. Leaf samples taken from the germinated seedlings were genotyped to test whether they are in the 1:2:1 wildtype, heterozygous and homozygous are in Hardy-Weinberg equilibrium. If homozygous individuals are present in the germinated seedlings, they should be in a 1:2 ratio compared to the heterozygous individuals: If homozygous Pro-197-Thr mutations are lethal, all of the resistance should be endowed by heterozygous Pro-197-Thr mutations (Table 5.8). Only one homozygous individual was identified for GBR_12105 cross 10 (Table 5.11) - there was no obvious morphological difference between this homozygous seedling and the others. Across all crosses, the total wildtype, heterozygous and homozygous ratio was 8:13:1. This is a further indication of lethality associated with homozygous Pro-197-Thr mutations, although with one homozygous individual identified, a small number of homozygous Pro-197-Thr mutations may be viable.

Table 5.8: Zygosity of germinated seed from the germination experiment. All of the crosses exhibited a significantly different (Fisher's exact test $P < 0.05$) ratio to the 1:2:1 wildtype, heterozygous and homozygous ratio expected if germinated seeds abide by Hardy-Weinberg equilibrium.

Population	Cross	Number of samples	Wildtype	Heterozygous	Homozygous
	6	2	1	1	0
GBR_12105	10	10	5	4	1
	12	12	4	8	0
	2	25	9	16	0
GBR_12075	9	13	6	7	0
	10	23	7	16	0
Total		85	32	52	1

5.4.4 Modelling the affect of lethal homozygous Pro-197-Thr mutations

Two competing models - a model in which homozygous Pro-197-Thr mutations do not exhibit lethality, and a model in which homozygous Pro-197-Thr mutations are lethal - were parameterized and implemented to predict the affect that lethal homozygous Pro-197-Thr mutations have on the evolution of ALS herbicide resistance in *A. myosuroides.* Initially (0–3 years), the evolution of Pro-197-Thr target-site resistance within the seed produced at reproduction is identical between lethal and non-lethal homozygous Pro-197-Thr mutation models (Figure 5.3). After three years, the proportion of ALS resistant seed produced at reproduction in the lethal and non-lethal homozygous Pro-197-Thr mutations models begins to diverge. In the lethal homozygous Pro-197-Thr mutation model, the proportion of the seed produced at reproduction exhibiting ALS resistance asymptotes at 0.5 in year 5: In the non-lethal homozygous Pro-197-Thr mutation model, the proportion of seed produced at reproduction exhibiting ALS resistance reaches 1.0 in year 10 (Figure 5.3).

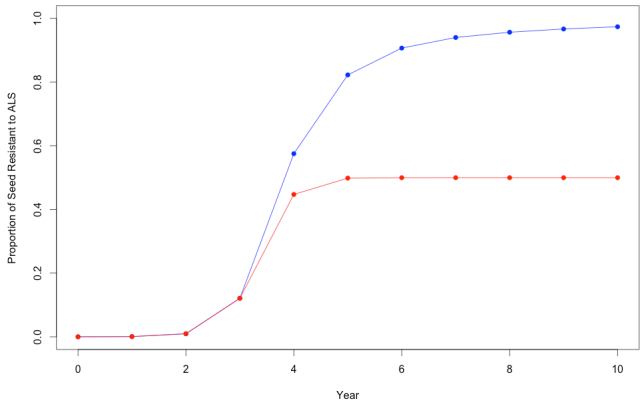


Figure 5.3: The proportion of plants modelled to be resistant via ALS target-site resistance under the scenarios in which homozygous Pro-197-Thr ALS target-site mutations are non-lethal (blue), and in which homozygous Pro-197-Thr ALS target-site mutations endow lethality (red). The model was run over a ten-year period under a management of continuous winter wheat and continuous application of and ALS herbicide.

When homozygous Pro-197-Thr mutations are non-lethal, the frequency of heterozygous Pro-197-Thr mutations in the seed produced at reproduction increased to 50% in year 5, before steadily decreasing as homozygous Pro-197-Thr mutations increased (Figure 5.4). When homozygous Pro-197-Thr mutations are lethal, heterozygous Pro-197-Thr mutations within the seed produced at reproduction reach a frequency of 0.5 in year 5 and remain at this frequency (Figure 5.4). The model predicts that when homozygous Pro-197-Thr mutations are lethal, the ratio heterozygous: wildtype genotype ratio within seed produced at reproduction will be 2:1 (Figure 5.4), a ratio not significantly different to that identified experimentally (Table 5.7).

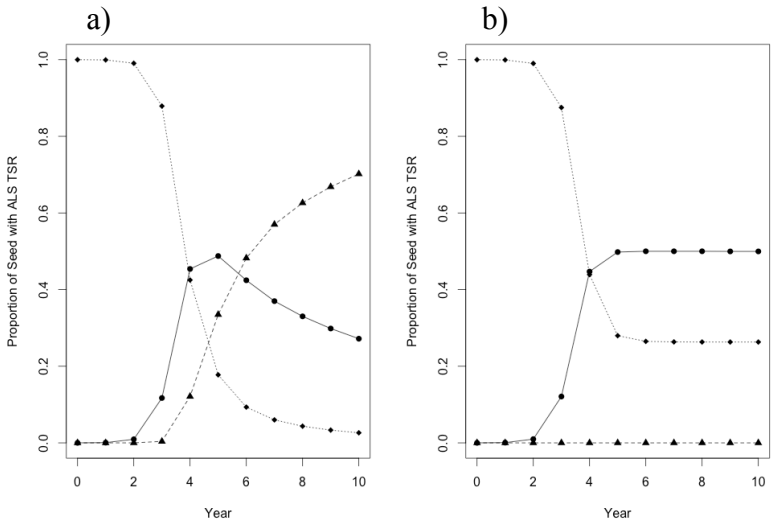


Figure 5.4: The proportion of plants modelled to be resistant via heterozygous and homozygous ALS target-site resistance under the scenarios in which homozygous Pro-197-Thr ALS target-site mutations are non-lethal (a), and in which homozygous Pro-197-Thr ALS target-site mutations endow lethality (b). The proportion of individuals modelled as possessing wildtype (diamonds), heterozygous (circle) and homozygous (triangle) Pro-197-Thr ALS target-site mutations are shown. Wildtype, heterozygous and homozygous proportions sum to 1 in (a), while in (b) they only sum to 0.75 as the homozygous proportion remains at 0 due to homozygous lethality.

5.5 Discussion

5.5.1 Segregation of Pro-197 individuals of seed produced from crosses

Under the principle of Mendelian inheritance (detailed in section 5.1.3), it is anticipated that progeny produced from crossing two heterozygous individuals will have a wildtype: heterozygous: homozygous allelic ratio of 1:2:1, and a phenotypic frequency of resistance of 3:1. The phenotypic ratio of resistance: susceptibility observed in progeny produced from three paired heterozygous Pro-197-Thr crosses for each of two *A. myosuroides* crosses is approximately 1.6:1, a ratio that is not significantly different to the 2:1 survival: mortality ratio expected if homozygous individuals are lethal. Similarly, genetic analysis of Pro-197-Thr individuals from phenotyping and germination tests revealed wildtype: heterozygous: homozygous

allelic ratios of 1: 1.6: 0 and 8: 13: 1 respectively; again, these ratios are not significantly different from the 1:2:0 wildtype: heterozygous: homozygous allelic ratio expected if homozygous individuals are recessively lethal.

The genotyping of leaf samples from the germination test gave no indication to the cause of the absent homozygous Pro-197-Thr mutation. If homozygous Pro-197-Thr mutations had been identified in the genotyped leaf samples from the germination test in large numbers, it may have been concluded that homozygous individuals germinate, but do not survive as a result of a fitness penalty imposed by the homozygous Pro-197-Thr mutation. As only one homozygous Pro-197-Thr mutation was identified, this appears not to be the case. To identify whether the absence of homozygous Pro-197-Thr mutations are a pre-zygotic or post-zygotic effect, further work is needed in the form of the genotyping of un-germinated seed. If individuals possessing homozygous Pro-197-Thr mutations are present in the un-germinated seed, this is an indication that seeds with the mutations are present but do not germinate (a post-zygotic lethal effect); If individuals possessing homozygous Pro-197-Thr mutations are not present in the un-germinated seed, this is an indication that seeds with the mutations do not mature and are therefore not present (a pre-zygotic lethal effect). Elucidating whether the absence of homozygous Pro-197-Thr mutations is as a result of a pre-zygotic or post-zygotic affect may not be possible, due to the small amount of DNA present in the seed of *A. myosuroides,* and there being different ploidy of the embryo (2N) and endosperm (3N), which will complicate DNA extraction (Beffa, personal communication).

By modelling the affect that a lethal homozygous Pro-197-Thr mutation will have on the evolution ALS target-site resistance in an *A. myosuroides* population, it can be seen that the initial rate of selection (0-3 years) will not be affected, as heterozygous mutations account for the target-site resistance during this period in both scenarios. When homozygous Pro-197-Thr mutations are non-lethal, the frequency of heterozygous Pro-197-Thr mutations in the new seed increased to 0.5 in year 5, before steadily decreasing as homozygous Pro-197-Thr mutations increased. As the population is under constant ALS selection (so not at Hardy-Weinberg equilibrium), heterozygous individuals decrease as homozygous individuals are selected to fixation. However over longer time periods, lethal homozygous Pro-197-Thr mutation limit the proportion of Pro-197 target-site resistant individuals to 0.5, as Pro-197 target-site resistance can only be endowed by heterozygous individuals. The model predicts that when homozygous Pro-197-Thr mutations are lethal, the ratio heterozygous: wildtype genotype ratio will be 2:1, a ratio not significantly different to that identified experimentally as discussed above.

5.5.2 Segregation of Pro-197 in previous studies

This is the first piece of research specifically exploring the segregation and inheritance of Pro-197-Thr ALS mutations in *A. myosuroides*. However, there has been one publication that has alluded to, but not formally tested, this phenomenon. During the characterisation of ALS resistant *A. myosuroides* in the UK, Marshall and Moss (2008) noted that all individuals (of 42 mesosulfuron-methyl + iodosulfuron-methyl-sodium resistant samples taken from seven populations) were heterozygous for the Pro-197-Thr mutation. The heterozygosity of these Pro-197-Thr mutations was confirmed in one population by cloning the ALS gene into plasmids and

sequencing. This identification of only heterozygous Pro-197-Thr by Marshall and Moss (2008) is contradicted by studies identifying homozygous Pro-197-Thr mutations in populations of *Lolium rigidum* (Collavo and Sattin 2014), *Papaver rhoeas* (Délye *et al* 2011) and *Alopecurus myosuroides* (Marshall *et al* 2013).

The discrepancy between this work, the Marshall and Moss (2008) study, and the studies identifying homozygous Pro-197-Thr mutations (Délye *et al* 2011; Marshall *et al* 2013; Collavo and Sattin 2014), may be as a result of the time since initial selection of the Pro-197-Thr mutations within the populations studied. The resistance endowed by Pro-197-Thr target-site mutations may be older within the populations exhibiting homozygous Pro-197-Thr mutations; potentially enabling secondary mutations/mechanisms to occur that counteract the lethality of homozygous Pro-197-Thr target-site mutations identified here. The one homozygous plant identified from the germination test (Table 5.11) may be evidence of the emergence of homozygous Pro-197-Thr by this method.

5.5.3 Potential causes of homozygous Pro-197-Thr lethality in A. myosuroides

An absence of Hardy-Weinberg equilibrium within a population can be caused by a number of factors. For example, segregation distortion and meiotic drive are phenomena that increase the frequency of a particular allele within the progeny to more than the 50% expected under Mendelian inheritance (Kozielska *et al* 2010). Segregation distortion can have detrimental effects on fitness. Homozygous distorter genes have been found can cause sterility and even lethality in *Mus musculus* and *Drosophila melanogaster* individuals (Burt and Trivers, 2006). Here however - due to the almost complete failure to identify homozygous Pro-197-Thr mutations and

the absence of any evidence of segregation distortion - it is considered that individuals homozygous for the Pro-197-Thr mutation are recessively lethal. An increase or decrease in the activity of ALS enzymes conferred by homozygous Pro-197-Thr mutations may contribute to the homozygous lethality seen in these experiments. Li *et al* (2013) studied the activity of ALS target-site mutations in *Raphanus raphanistrum* (wild radish), identifying an increase in extractable ALS activity for Ala-122-Tyr, Pro-197-Ser, Asp-376-Glu mutations, and a decrease in extractable ALS activity for Trp-574-Leu mutations. A decrease in ALS activity associated with homozygous Pro-197-Thr may lead to a fatally low level of production of the essential amino acids leucine, isoleucine and valine. Conversely, increased activity can lead to the overproduction of ALS end products (leucine, isoleucine and valine), either to the detriment of other resources, or to a level that they become toxic. Valine, an end product of the ALS metabolic pathway, has been shown to be toxic to the germination of *Arabidopsis thaliana* if found in too high concentration (Dündar 2004). Fitness costs associated with factors not directly associated with homozygous Pro-197-Thr resistance (e.g. pollen tube growth or pollen being less or not fertile compared to the wild type) may also be a possible cause of the observed homozygous lethality.

5.6 Conclusions

To answer the question whether homozygous Pro-197-Thr mutations are lethal, or at least not in Hardy-Weinberg equilibrium or not, phenotyping, germination, and genotyping experiments were conducted on individual plants produced from controlled crosses of heterozygous Pro-197-Thr individuals. Phenotypically, the crosses closely resembled the 2:1 survival: mortality expected if homozygous Pro-

197-Thr target-site mutations are lethal. Only one homozygous Pro-197-Thr plants was identified throughout the experiment, with the ratio of wildtype: heterozygous: homozygous plants identified being approximately 1: 2: 0. The results of this research imply that homozygous Pro-197-Thr target-site mutations are not found in Hardy-Weinberg equilibrium, as a result of a lethality associated with the mutation when in the homozygous state. Simulation models have shown that the absence of homozygous Pro-197-Thr mutation within *A. myosuroides* populations will limit the proportion of Pro-197 target-site resistant individuals to 0.5, as Pro-197 target-site resistance can only be endowed by heterozygous individuals. Further work, involving the genotyping of un-germinated seed and pollen grains, is required to identify whether homozygous lethality is due to a pre-zygotic or post-zygotic lethal effect.

6.0 Establishing the effect of pre-existing ACCase enhanced metabolism on the rate of selection of ALS enhanced metabolism in *Alopecurus myosuroides.*

6.1 Introduction

6.1.1 Resistance endowed by enhanced metabolism in A. myosuroides

Enhanced metabolism (EMR) is a mechanism of herbicide resistance whereby enzymes detoxify the active herbicide into inert compounds (Délye 2012). With it estimated that ACCase EMR is present in 75% of plants from 243 French *A. myosuroides* populations (Délye *et al* 2011), 80% of 53 Danish *A. myosuroides* populations (Keshtkar *et al* 2015), and every *A. myosuroides* population tested in the UK (Chapter 2), enhanced metabolism is undoubtedly an important resistance mechanism in *Alopecurus myosuroides.*

Enhanced metabolism is a polygenic trait. It is speculated that EMR can evolve from recurrent selection and recombination of standing genetic variation in stress responsive pathways (although the importance of standing genetic variation vs. *de novo* mutations in the selection of NTSR is still unclear) (Petit *et al* 2010; Délye *et al* 2013; Neve *et al* 2014). So far, only one gene involved in *A. myosuroides* enhanced metabolism is known. *AmGSTF1* - while not directly involved in metabolization - appears to regulate a plants ability to detoxify the PSII inhibiting herbicides atrazine (chloro-s-triazine) and chlorotoluron (phenylurea) (Cummins *et al* 2013). With the exact genes involved in enhanced metabolism not yet determined, it has been observed as few as three loci could combine to endow metabolic resistance (Petit *et*

al 2010). Further observations have noted that the minimum number of loci needed to endow enhanced metabolism could be different for each mode of action, e.g. chlorotoluron (1 loci), fenoxaprop-p-ethyl (1 or 2 loci), pinoxaden (2 loci), mesosulfuron + iodosulfuron (2 loci), flufenacet (between 1 and 3 loci) (Rosenhauer *et al* 2015).

In separate experiments, Petit *et al* (2010) and Rosenhauer *et al* (2015) investigated the polygenic nature and segregation of enhanced metabolism in *A. myosuroides*. They both concluded that at least one of the genes endowing enhanced metabolism of the aryloxyphenoxypropionate ACCase inhibitor fenoxaprop-p-ethyl confers cross-resistance to pinoxaden, an ACCase inhibitor of the class phenlypyrazoline. It has been suggested that genes conferring enhanced metabolism to aryloxyphenoxypropionate ACCase inhibitors may also confer a degree of "pre-adaptation" to ALS modes of action (Délye *et al* 2011). Using four *A. myosuroides* populations experimentally selected with ALS herbicide - three with previously identified ACCase enhanced metabolism and no ALS enhanced metabolism, and one that had no enhanced metabolism to either mode of action - and dose response analysis, this chapter aims to address the hypothesis that enhanced metabolism to ACCase inhibitors also confers a degree of "pre-adaptation" to ALS modes of action.

6.1.2 Use of selection experiments in resistance research

The evolution of resistance to herbicides occurs over large temporal and spatial scales, making detailed long-term studies into resistance evolution impractical to conduct (Renton *et al* 2014). Therefore, to gain an understanding of the dynamics of resistance evolution, the response of populations to recurrent selection in glasshouse

experiments has been an invaluable source of information. *Lolium rigidum* populations selected at sub-lethal doses have shown that metabolic resistance can be rapidly selected over a limited number of generations (Neve and Powles 2005; Busi and Powles 2009; Manalil *et al* 2011; Busi *et al* 2012). This information has benefited resistance management, highlighting that cutting herbicide dose can increase the risk and rate of metabolism based resistance evolving (Neve *et al* 2014).

6.1.3 Dose-response analysis

Dose-response analysis is an essential analytical tool in weed science. Dose-response analysis quantitatively determines a population's response to a particular stress (Seefeldt *et al* 1995). In the context discussed here, the stressor is a chemical herbicide (with a specific mode of action), and the response is the effect that the herbicide has on the weed species in question. Dose-response analysis is used widely in agrichemical research, both to quantify the efficacy of new modes of action and to monitor the evolution of herbicide resistance.

To quantify the level of herbicide resistance within a population using dose-response analysis, it is required that a weed population is experimentally treated with a series of increasing herbicide doses. The plant response chosen to measure resistance can take many forms, e.g. fresh weight, dry weight, mortality, or fecundity. Regardless of the measure used, plant responses to a herbicide dose series are usually non-linear, hence, non-linear regression, assuming one of a number of underlying distributions (logistic, log-logistic, or Weibull distribution) is used for analysis (Seefeldt *et al* 1995). Using non-linear regression, parameters such as the populations ED_{50} (the dose at which the response is half of that of an untreated control) can be determined.

By comparing the parameters estimated from the non-linear regression to that of a known susceptible and/or resistant standard, the level of resistance within the population can be quantified.

6.2 Objectives

The work reported in this chapter aims to determine if pre-existing ACCase enhanced metabolism impacts upon the rate of selection ALS enhanced metabolism in experimental evolutionary studies. Four *A. myosuroides* populations were used; three with previously identified ACCase enhanced metabolism and no ALS enhanced metabolism, and one that had no enhanced metabolism to either mode of action. After each population had undergone two generations of low-dose selection with the ALS inhibiting herbicide mesosulfuron-methyl + iodosulfuron-methyl-sodium, the hypothesis that pre-existing ACCase enhanced metabolism has an effect on the selection of ALS enhanced metabolism, and therefore the rate of ALS enhanced metabolism selection is greater in the populations with pre-existing ACCase enhanced metabolism was tested.

6.3 Materials and methods

6.3.1 Plant material

Four populations of *Alopecurus myosuroides* were used - identified throughout as BR, 12135, 6001 and WE respectively. These populations were chosen based on previously identified characteristics of ALS and ACCase resistance (Table 6.1). These populations were exposed to two rounds of recurrent selection with low doses of the ALS herbicide mesosulfuron-methyl sodium iodosulfuron-methyl-sodium (as described in sections 6.3.2 – 6.3.5 and Figure 6.2) before dose response and

herbicide metabolite experiments were conducted to compare selected and unselected lines.

Table 6.1: A description of the populations used throughout the selection experiment and their ALS and ACCase resistance characteristics

Population	Resistance characteristics of the populations
BR	• A known ALS and ACCase susceptible standard population
12135	• A population collected from the UK in 2012 • No ALS resistance, EMR or TSR • ACCase resistance endowed via EMR only (no TSR)
6001	• A standard ACCase EMR resistant population • No ALS resistance, EMR or TSR • ACCase resistance endowed via EMR only (no TSR)
WE	• An ACCase EMR resistant population that had EMR selected from a susceptible population using low doses of fenoxaprop over two generations in a glasshouse (Lynch PhD thesis 2014) • No ALS resistance, EMR or TSR • ACCase resistance endowed via EMR only (no TSR)

6.3.2 First round of selection

For each population by herbicide dose combination listed below, 10 6" pots containing 10 plants were established by double sowing two seeds into ten evenly spaced locations. For each herbicide dose combination, two additional populations, ALOMY_GBR_12111 (a population collected in 2012 and identified as having low levels of ALS phenotypic resistance and low levels of ALS enhanced metabolism (fully described in chapter 3, Table 3.11), and ALOMY_GBR12066 (a population collected in 2012 and identified as having high levels of ALS phenotypic resistance endowed by enhanced metabolism (fully described in chapter, 3 Table 3.1)), were included as a negative and positive control respectively to test the efficacy of herbicide application. Pots were arranged in a completely randomised block design with two pots for each treatment included in each of five blocks. Pots were filled

with a 2:1:1 mix of J. Arthur Bower's topsoil (English loam blended with organic matter and nutrients, pH: 6.5 – 7.5), 0.5 Levington growing media: M2 (pH: 5.5 – 6, N: 200, P: 150, K: 200 mg/liter), and 0.25 J. Arthur Bower's silver sand (lime-free washed silica sand) and placed in a glasshouse compartment (22^{o}C day/16^{o}C night; 14 hour photoperiod). Plants were watered as required. Three weeks after sowing, germinated seedlings were thinned to one plant at each sowing location.

Plants were treated with one of five doses below the recommended UK field rate (1x) of the ALS herbicide mesosulfuron-methyl (1x = 12 g ha^{-1}) + iodosulfuron-methyl-sodium (1x = 2.4 g^{ha-1}) and the adjuvant Biopower (0.27 kg a.i ha^{-1}); the five doses were: 0x, 0.1x, 0.2x, 0.4x, and 0.7x (Figure 6.2(a)). As the ALS resistance phenotype of the populations was not known in advance, plants were exposed to this range of doses to identify which would be most appropriate for low dose recurrent selection. Herbicide was applied using a Berthoud 2000 knapsack sprayer. The sprayer was set to apply herbicide at a pressure of 300 kPa while walking at a fixed pace of 3 kph. A spray volume of 200L/ha was delivered through a Hypro standard flat fan tip (110 degrees F110-03 ultra blue nozzle), positioned 40cm above the height of the modular tray. Plants were returned to glasshouse compartment and watered regularly after herbicide application. Three weeks after herbicide application, the number of surviving plants was determined.

6.3.3 Crossing of survivors from the first round of selection

A total of 10, 8 16 and 5 individuals surviving herbicide application for populations BR, 12135, 6001 and WE respectively were grown to maturity and bulk-crossed to produce a selected (F1S) generation (Table 6.2, Figure 6.2(b-c)). These survivors

were taken from doses 0.2x and 0.4x for populations 12135, 6001 and WE, and 0.1x for BR (Table 6.2). These doses were used as they represented the most resistant individuals for each population. Survivors from more than one dose were used for the bulk crossing of populations 12135, 6001 and WE, due to the low survival at each individual dose; for population BR, 0.1x was the only dose that exhibited any survival (Table 6.2). The bulk-crossing of populations took place within a galas leys compartment; a polythene tunnel containing pollen proof cages that segregate individual selection lines to enable cross-pollination between selected plants and prevent cross-pollination between selection lines (Figure 6.1).

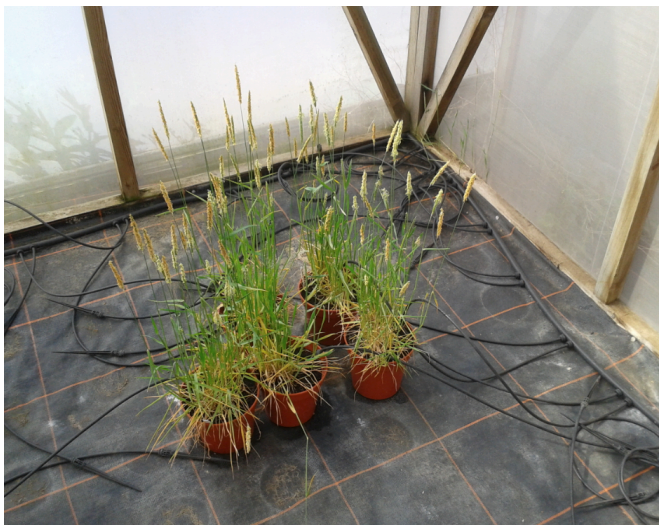


Figure 6.1: Flowering *A. myosuroides* plants within a galas leys compartment.

From the untreated (0x) control dose of each population, 10, 8 16 and 5 individuals for populations BR, 12135, 6001 and WE (the same number of individuals bulk crossed to produce the selected lines) were bulk crossed to create an F1 untreated control (F1C) generation (Table 6.2, Figure 6.2(c)). In July and August as seed matured, it was harvested from each cross as a bulked seed sample and stored in paper bags in a drying room (relative humidity = 15%) until use.

Table 6.2 Number of plants used to create the next generation of seed for each population's selected and untreated lines. Numbers highlighted in red are the total number of plants taken forward for seed production. The numbers in brackets = the number of plants taken from each dose of mesosulfuron-methyl + iodosulfuron-methyl-sodium (x).

Population	Selected (F1S)	Untreated (F1C)
BR	10 (10 at 0.1x)	10
12135	8 (6 at 0.2x, 2 at 0.4x)	8
6001	16 (5 at 0.2x, 11 at 0.4x)	16
WE	5 (3 at 0.2x, 2 at 0.4x)	5

6.3.4 Second round of selection

The F1S seed generation was selected again using the same experimental design (detailed in section 6.3.2) (Figure 6.2(d)). Each F1U seed generation (with 10 6" pots containing 10 plants of one populations F1U) was also included within the completely randomised block experimental design (detailed in section 6.3.2), to act as a control to demonstrate that any change in resistance was due to selection (Figure 6.2(d)).

6.3.5 Crossing of survivors from the second round of selection

As with the first round of selection (section 6.3.2), a number of surviving herbicide application for populations BR (5 survivors), 12135 (12), 6001 (13) and WE (18) were grown to maturity and bulk-crossed to produce a selected (F2S) generation in the galas lays (Table 6.3) (Figure 6.2(e-f)). These survivors were taken from doses 0.4x and 0.7x for populations 12135 and 6001, 0.1x and 0.2x for BR, and 0.7x for WE (Table 6.3). These doses were used as they represented the most resistant individuals for each population. Survivors from more than one dose were used for the bulk crossing of populations BR, 12135 and 6001, due to the low survival at each individual dose (Table 6.3). With survivors identified at the 0.7x dose for populations 12135, 6001 and WE, and the 0.2x dose for BR after the second

selection, there is an indication that there has been a response to selection during in the F1S generation, as survivors at these doses were not identified during the first round of selection. From the untreated (0x) control dose of each population, 5, 12,13 and 8 individuals for populations BR, 12135, 6001 and WE (the same number of individuals bulk crossed to produce the selected lines (Table 6.3)) were bulk crossed to create an F2 untreated control (F2U) generation (Table 6.3, Figure 6.2(f)). In July and August as seed matured, it was harvested from each cross as a bulked seed sample and stored in paper bags in a drying room (relative humidity = 15%) until use.

Table 6.3 Number of plants used to create the next generation of seed for each population's selected and untreated lines. Numbers highlighted in red are the total number of plants taken forward for seed production. The numbers in brackets = the number of plants taken from each dose of mesosulfuron-methyl + iodosulfuron-methyl-sodium (x).

Population	Selected (F2S)	Untreated (F2C)
BR	5 (4 at 0.1x, 1 at 0.2x)	5
12135	12 (5 at 0.4x, 8 at 0.7x)	12
6001	13 (6 at 0.4x, 8 at 0.7x)	13
WE	18 (18 at 0.7x)	18

6.3.6 Dose-response analysis

Original, F1S, F2S, and F2U seed lines for each population were assessed in a dose-response experiment to determine if there had been a response to selection with mesosulfuron-methyl-sodium + iodosulfuron-methyl. Seeds were germinated in petri dishes containing 2 sheets of Whatman No. 1 filter paper plus 5 ml of 2g/L KNO_3. Petri dishes were placed in an incubator (12hrs: 23°c, 12hrs: 9°c) and following two weeks incubation germinated seedlings were transplanted into 6" plant pots.

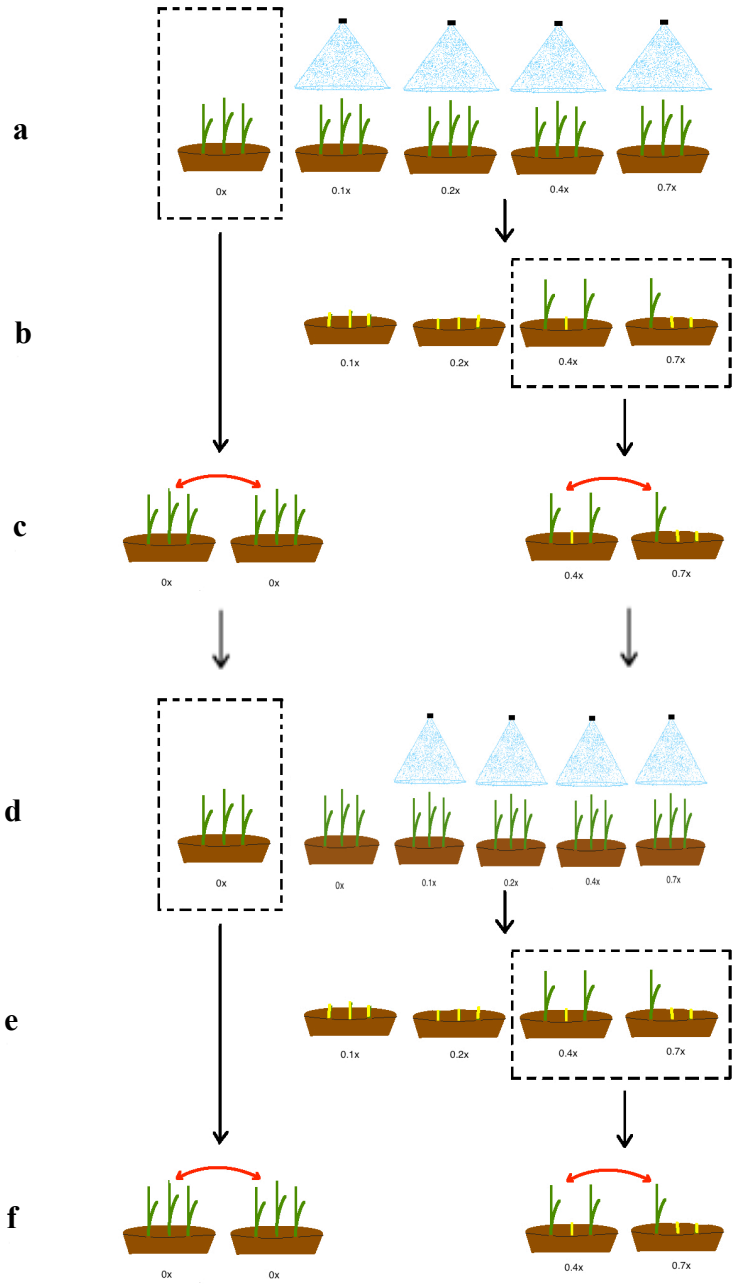


Figure 6.2: Selection protocol used (section 6.3.2 – 6.3.3) for each of the four experimental populations. (a) A set number of plants from each original population were exposed to two rounds of ALS selection by exposure to low doses - doses below the UK recommended field rate – of the herbicide mesosulfuron-methyl + iodosulfuron-methyl-sodium. Five doses below the recommended UK field rate (1x = mesosulfuron-methyl (12 g ha^{-1}) + iodosulfuron-methyl-sodium (2.4 g^{ha-1})) were used: 0x, 0.1x, 0.2x, 0.4x, and 0.7x. (b, c) Plants surviving from selected doses of herbicide were grown to maturity and bulk crossed to create the selected F1 generation (F1S) for use in step d. Untreated (0x) plants were grown to maturity and bulk crossed separately to create an F1 control (F1C) line. (d - f) Seed of the selected F1 generation was selected as per steps a – c to create a selected F2 generation (F2S). Seed of the F1 untreated line was grown without selection (d) before bulk crossing (f) to create an untreated F2 line (F2C).

Each 6-inch pot (filled with a soil mix described in section 4.3.3.1) contained 10 seedlings of one generation (either original, F2C, F1S or FS2) of one population. The experimental set up encompassed three replicates arranged in a completely randomized block design. In each replicate there was one pot for each populations original, F1S, F2S, and FSC seed line (plus GBR_12111 and GBR12066 as a negative and positive control respectively to test the efficacy of herbicide application (see section 6.3.2)) at each of seven doses (0x, 0.05x, 0.1x, 0.2x, 0.4x, 0.8x, 1.6x) of mesosulfuron-methyl + iodosulfuron-methyl-sodium and the adjuvant Biopower (0.27 kg a.i ha^{-1}). Plants were grown in a glasshouse compartment (temperature: 22°c/16°c; day length: 14 hours with supplementary lighting) and watered regularly; fertiliser was applied when required. At the two leaf stage, the seven doses of mesosulfuron-methyl + iodosulfuron-methyl-sodium were applied (method described in section 6.3.3). Twenty-one days after herbicide application, the response of the plants was assessed as alive or dead, and proportion survival per replicate pot calculated.

6.3.7 Assessing mesosulfuron metabolites using HPLC

The metabolism of radiolabelled mesosulfuron was compared between the original and F2S generation of each of the four populations to determine if there was an increase in the level of mesosulfuron metabolism following selection. 36 plants of each population's original and FS2 generation were grown and maintained in a glasshouse under the conditions described in chapter 2 (section 2.3.2). Four weeks after sowing, leaf samples were taken from the thirty-six plants from each of the four original and F2S populations and HPLC analysis of mesosulfuron metabolites was performed (detailed in section 2.3.4).

6.3.8 Statistical analysis

6.3.8.1 Dose-response analysis of survival data

The survival data for each population was analysed using dose-response analysis in the drc package of R (Ritz and Streibig 2011; R Development Core Team 2012). The best fitting model was applied to each population. To the original, F1U, F2U, and F2S generations of population BR, a 3-parameter logistic model was fitted (Equation 6.1):

$$f(x) = d / (1+(x/e)^{(-b)})$$

(Equation 6.1)

Where d is the upper asymptote, the lower asymptote is 0, the slope (b) of the model set to 1, and e is the ED_{50} (mesosulfuron-methyl + iodosulfuron-methyl-sodium dose causing 50% mortality). No model could be fitted to population BR's F1S generation. A 2-parameter log-logistic model was fitted to all generations (original, F2U, F1S, and F2S) of populations 12135 and 6001 (equation 6.2):

$$f(x) = 1 / (1 + \exp(b(\log(x) - e)))$$

(Equation 6.2)

Where the upper asymptote is set at 1, the lower asymptote is set at 0, b is the slope of the model, and e is the ED_{50}. Finally, to the original, F2U and F2S generations of population WE, a 2-parameter Weibulls.2 model was fitted (equation 6.3):

$$f(x) = c + (d - c)(1 - \exp(-\exp(b(\log(x) - \log(e)))))$$

(Equation 6.3)

Where the upper asymptote (c) is set at 1, the lower asymptote (d) is set at 0, b is the slope of the model, and e is the ED_{50}. No model could be fitted to population WE's

F1S generation. From these models, the ED_{50} and ED_{90} (mesosulfuron-methyl + iodosulfuron-methyl-sodium dose causing 90% mortality) values were calculated for each generation/population. The resistance index (Equation 6.4) for each selected (F2U, F1S, F2S) generation was tested for significance to the original generation using a Students t-test.

$$LD_{value} \text{ of selected generation} / LD_{value} \text{ of original population} \quad \text{(Equation 6.4)}$$

6.3.8.2 Statistical analysis of metabolite data

A boxplot was produced for each population's original and FS2 seed lines to visually assess the spread of the data. Each population's FS2 seed generation was compared to its respective original using a one-way analysis of variance (ANOVA) and Tukey's HSD. All data was analysis was performed using R (R Development Core Team 2012).

6.4 Results

6.4.1 Dose-response analysis of survival

The dose response analysis conducted on the original, F2C and F2S generations of population BR shows a small non-significant ($P > 0.05$) increase in ED_{50} (original = 0.30 (0.26), F2S = 0.71 (0.13)) values between the original and F2S; a significant increase in ED_{90} (original = 0.39 (0.28), F2S = 1.15 (0.20)) values between the original and F2S generations was observed (Figure 6.3, Table 6.4). There was no significant ($P > 0.05$) change in ED_{50} (F2C = 0.43 (0.19)) and ED_{90} (F2C = 0.55 (0.08)) values between the original and F2C control (Figure 6.3, Table 6.4). For

population 12135, there was a significant ($P < 0.05$) increase in ED_{50} value observed in all generations (F2C, F1S, and F2S) when compared to the original (Figure 6.3, Table 6.4), but only a significant ($P < 0.05$) increases in ED_{90} values in the selected (F1S and F2S) lines (Figure 6.3, Table 6.4).

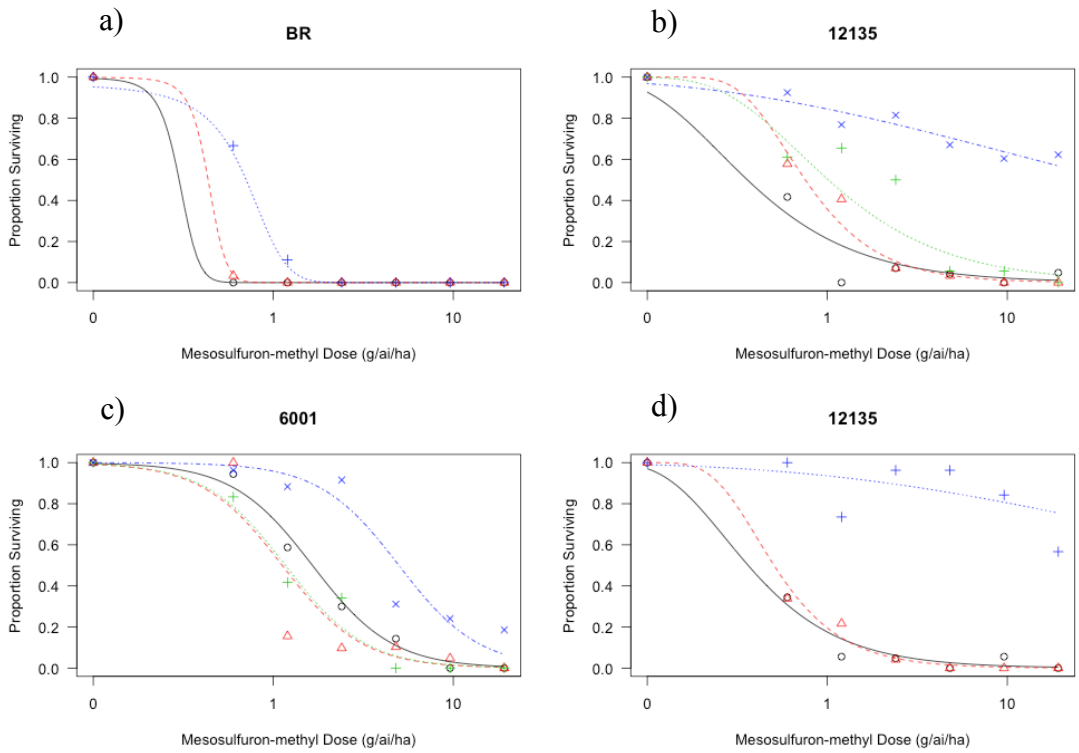


Figure 6.3: Dose-response models fitted to the original, F1 selected (F1S), F2 control (F2C), and F2 selected (F2S) of the four populations – (a) BR, (b) 12135, (c) 6001 and (d) WE. The black line represents each population the original generation, the red line represents the F2 control (F2C) generation, the green line represents the F1 selected (F1S) generation, and the blue line represents the F2 selected (F2S) generation. Points represent the mean proportion survival for each population/dose combination. All models fitted are as indicated in section 6.3.8.1.

The F2S generation was the only generation of population 6001 that exhibited a significant ($P < 0.05$) increase in ED_{50} (original = 1.65 (0.21), F2S = 4.99 (0.60)) and ED_{90} (original = 5.08 (0.78), F2S = 15.34 (2.43)) values when compared to the original (Figure 6.3, Table 6.4). The ED_{50} and ED_{90} values for the F2C and F1S

generations of population 6001 were both non-significantly ($P > 0.05$) lower than the original population (Figure 6.3, Table 6.4). Likewise, the F2S generation for population WE was the only one exhibiting a significant ($P < 0.05$) increase in ED_{50} (original = 0.36 (0.16), F2S = >19.20 ($9*10^2$)) and ED_{90} (original = 1.61 (0.50), F2S = >19.20 ($1*10^7$)) values when compared to the original (Figure 6.3, Table 6.4). The F2C generation exhibited no significant ($P > 0.05$) change in either ED_{50} or ED_{90} (Figure 6.2, Table 6.4).

Table: 6.4: ED_{50} (+/- standard error) and ED_{90} (+/- standard error) for the original, F2 control (F2C), F1 selected (F1S), and F2 selected (F2S) generations for populations BR, 12135, 6001 and WE. All models fitted are as indicated in section 6.3.8.1. ED_{50} and ED_{90} values indicated by >19.20 (-) are those that are above the highest tested dose of 19.20 g a.i. ha^{-1}, therefore their standard errors are not shown.

Population		ED_{50} Value in g a.i. ha^{-1}	P-value	ED_{90} Value in g a.i. ha^{-1}	P-value
BR	Ori	0.30 (0.26)	-	0.39 (0.28)	-
	F2C	0.43 (0.19)	0.6500	0.55 (0.08)	0.5667
	F2S	0.71 (0.13)	0.1321	1.15 (0.20)	0.0071*
12135	Ori	0.35 (0.19)	-	2.21 (0.76)	-
	F2C	0.76 (0.09)	0.044*	2.46 (0.54)	0.022
	F1S	1.01 (0.21)	0.001*	6.87 (3.07)	0.000*
	F2S	>19.20 (34.48)	0.000*	>19.20 ($1*10^5$)	0.000*
6001	Ori	1.65 (0.21)	-	5.08 (0.78)	-
	F2C	1.16 (0.14)	0.097	3.60 (0.56)	0.097
	F1S	1.12 (0.16)	0.089	3.45 (0.52)	0.089
	F2S	4.99 (0.60)	0.000*	15.34 (2.43)	0.000*
WE	Ori	0.36 (0.16)	-	1.61 (0.50)	-
	F2C	0.51 (0.11)	0.428	1.53 (0.35)	0.892
	F2S	>19.20 ($9*10^2$)	0.000*	>19.20 ($1*10^7$)	0.000*

6.4.2 Analysis of herbicide metabolites

The amount of mesosulfuron metabolised by each populations original and FS2 generation was plotted as a boxplot (Figure 6.4). Population BR exhibited no increase in median between the original and FS2 generation after two years of selection; however, a there was an increase in upper quartile from 6.77 (original) to

9.16 (FS2). One individual - represented as an outlier (Figure 6.4) - within the BR FS2 generation exhibited a large (42.86) percentage of mesosulfuron metabolization relative to both original and the rest of the F2S generations.

The median of population 12135 approximately doubled, from 7.46 in the original generation to 14.89 in the F2S generation after two years of selection, as did the upper quartile from 12.62 (original) to 21.39 (F2S) (Figure 6.4). Six outlying individuals in the 12135 F2S generation, with mesosulfuron metabolization values ranging from 56 – 65%, exhibited a large increase in mesosulfuron metabolization when compared to the original generation (Figure 6.4).

Population 6001 exhibited no large increase in median (original = 14.60, F2S = 16.39) or upper quartile (original = 20.75, F2S = 20.52) between the original and FS2 generation after two years of selection. Five outlying individuals in the 6001 F2S generation, with mesosulfuron metabolization values ranging from 54 – 63%, exhibited a large increase in mesosulfuron metabolization when compared to the original generation (Figure 6.4).

An ANOVA and Tukey's HSD was used to test whether the changes in mesosulfuron metabolism identified between the original and FS2 generation in each of the four populations is significant (Table 6.5). Only populations 12135 and WE showed a significant increase in the mean amount of mesosulfuron metabolised between the original and FS2 populations (Table 6.5).

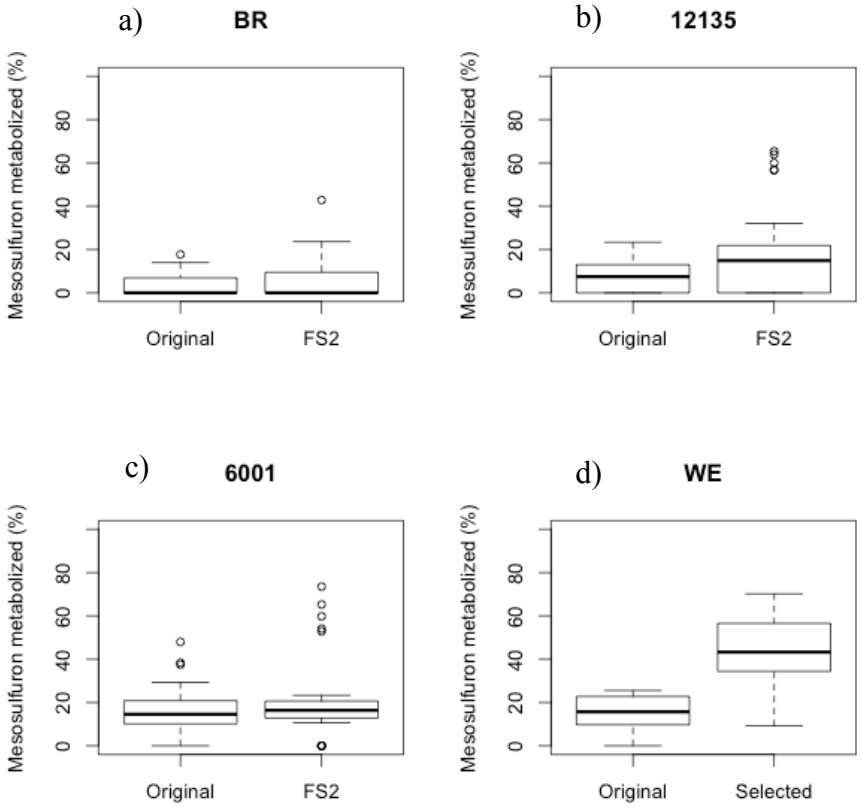


Figure 6.4: Amount of mesosulfuron metabolised (%) in for each population's ((a) BR, (b) 12135, (c) 6001, (d) BR) Original and FS2 generation.

Table 6.5: Change in mean amount of mesosulfuron metabolised between the original and FS2 generations for each population. The mean amount of mesosulfuron metabolised (+/- standard error of the mean) is represented for each populations Original and FS2 generation. P-values are those taken from the Tukey's HSD analysis. P-values marked with an asterisk (*) indicate that the difference between the original and FS2 generation is significant ($P < 0.05$).

Population	Generation	Mean amount of mesosulfuron metabolised (+/- SE_M)	P-Value
BR	Original FS2	3.81 (0.98) 6.68 (1.95)	0.999
12135	Original FS2	6.81(1.15) 18.59 (3.21)	0.041*
6001	Original FS2	16.15 (1.71) 20.63 (3.00)	0.999
WE	Original FS2	14.82 (1.38) 44.17 (2.69)	0.000*

6.5 Discussion

6.5.1 Comparing the rate of ALS enhanced metabolism selection in populations with and without pre-existing ACCase enhanced metabolism

Four *A. myosuroides* populations - three (12135, 6001, WE) previously identified as having ACCase enhanced metabolism and no ALS enhanced metabolism, and one (BR) that had no enhanced metabolism to either mode of action inhibitors – were selected over two generations with the ALS herbicide mesosulfuron-methyl + iodosulfuron-methyl-sodium to address the hypothesis that enhanced metabolism to ACCase inhibitors also confers a degree of "pre-adaptation" to ALS modes of action. Dose-response analysis of survival revealed that populations possessing pre-existing ACCase enhanced metabolism (12135, 6001, WE) exhibited significantly large increases in ED_{50} and ED_{90} after two years of selection, whereas the increases in ED_{50} and ED_{90} in the population with no pre-existing ACCase enhanced metabolism (BR) were relatively smaller (with the increase in ED_{90} only being significant). Likewise, greater increases in the proportion of mesosulfuron metabolised was observed after two years of selection in the populations with pre-existing ACCase enhanced metabolism – significantly so for populations 12135 and WE – when compared to the population without pre-existing ACCase enhanced metabolism. These finding is the first experimental proof of the hypothesised suggested by Délye *et al* (2011), that alleles conferring enhanced metabolism to aryloxyphenoxypropionate ACCase inhibitors may also confer a degree of "pre-selected" enhanced metabolic resistance to ALS modes of action.

6.5.2 Interaction between ALS and ACCase mechanisms of enhanced metabolism

Enhanced metabolism is a polygenic trait, speculated to evolve from recurrent selection and recombination of standing genetic variation in stress responsive

pathways (Petit *et al* 2010; Délye *et al* 2013; Neve *et al* 2014). The precise genes involved in ALS and ACCase enhanced metabolism in *A. myosuroides* are unknown, however it has been estimated that the minimum number of loci needed to endow enhanced metabolism to fenoxaprop-p-ethyl and mesosulfuron + iodosulfuron are 1 or 2, and 2 loci respectively, meaning could mean that there be many genes per locus (Rosenhauer *et al* 2015). Recently four genes (two P450 Cytochrome mono-oxygenases, one nitrogen mono-oxygenase, and a Glutathione sythanse) in *Lolium rigidum* and one (*AmGSTF1*) in *Alopecurus myosuroides* associated with the enhanced metabolism trait have been identified (Cummins *et al* 2013; Gaines *et al* 2014). Though not involved directly in metabolization, *AmGSTF1* appears to regulate a plants ability to metabolise PSII inhibiting herbicides atrazine (Cummins *et al* 2013): While the genes identified by Gaines *et al* (2014) aid in the expression of a metabolically derived diclofop resistant phenotype, and are induced by 2,4D, which confers diclofop resistance to sensitive plants when applied 24h before the herbicide.

From the HPLC analysis of mesosulfuron metabolization, it can be seen that the four populations studied exhibited disparate patterns of change in the level of mesosulfuron metabolized after two years of selection. In populations 12135 and 6001, six and five individuals respectively were able to metabolize a large percentage of mesosulfuron, while the majority of individuals remained at a lower level similar to that of the original. Contrastingly, the whole of population WE exhibited a significant shift towards higher levels of mesosulfuron metabolism, while only a small increase in mesosulfuron metabolism was seen in population BR. It is likely that the enhanced metabolism genes selected and inherited from each

population's standing genetic variation will play an important role in explaining the patterns observed.

Within populations with pre-existing ACCase enhanced metabolism, genes selected to endow enhanced metabolism to ACCase modes of action will be in high frequencies. If these genes also confer a degree of "pre-adapted" enhanced metabolism to ALS modes of action, then ALS metabolism will be selected more rapidly than in a population with no ACCase enhanced metabolism, as "pre-adapted" ALS metabolism genes will already be present in greater frequencies. The nature of the genes that confer but ACCase and "pre-adapted" ALS enhanced metabolism can only be speculated at this time, but it is possible they could be regulatory, similar to the *AmGSTF1* identified by Cummins *et al* (2013).

6.5.3 Implications for A. myosuroides management

The findings of this experiment potentially have implications for chemical management of *A. myosuroides* with ALS herbicides. If a population of *A. myosuroides* possess enhanced metabolism to and ACCase mode of action – which a large number of UK (Chapter 2) and European (Moss *et al* 2007; Delye *et al* 2007; Delye *et al* 2010; Hess *et al* 2012; Hull *et al* 2014; Keshtkar *et al* 2015) already do – then the rate at which ALS enhanced metabolism is selected may be increased compared to a population with no pre-existing ACCase enhanced metabolism. This puts pressure on existing chemical management regimes, as increased rates of ALS resistance evolution in *A. myosuroides* will lead to a quicker decrease in ALS herbicide efficacy. With this increased threat of resistance, as well as lack of new

MOA and existing MOA being removed from the commercial market, (Lutman *et al* 2013), a greater emphasis will be put on integrated weed management (IWM).

It must be noted however, how the ACCase enhanced metabolism "pre-adaptation" to ALS modes of action materialises in real time selection of ALS enhanced metabolism in the field is unknown. The affect in the field may be as observed here; it may be mitigated by other factors (e.g. environment), or even exacerbated in certain circumstances.

6.6 Conclusions

The work reported in this chapter aimed to determine if pre-existing ACCase enhanced metabolism within a population of *Alopecurus myosuroides* impacts upon the rate of ALS enhanced metabolism selection. After conducting a selection experiment over two generations - using three populations previously identified as having ACCase enhanced metabolism and no ALS enhanced metabolism, and one that had no enhanced metabolism to either mode of action - it was observed that populations possessing pre-existing ACCase enhanced metabolism exhibited increased rates of ALS enhanced metabolism selection. The implication for *A. myosuroides* is that accelerated rates of ALS enhanced metabolism selection may render the ALS modes of action ineffective sooner than expected, and reinforces the message to use all possible integrated weed management (IWM) practices to control *A. myosuroides* populations in order to keep the soil seed bank as low as possible.

7.0 Discussion

7.1 The presence and extent of Alopecurus myosuroides resistance in the UK

Arable weed control across the developed world is dominated by the application of herbicides (Naylor and Drummond 2002). The continuous application of a single mode of action (MOA) in a homogeneous environment offers an affordable and efficacious method of weed control, albeit unsustainable. The evolution of herbicide resistance is now a global issue, with 246 species of plant resistant to one or more of 157 herbicide active ingredients in 66 countries (Heap *et al* 2015).

The first aim of this thesis was to determine the presence and extent of post-emergent ALS and ACCase herbicide resistance in populations of *Alopecurus myosuroides* from an important arable region of the UK. Of the ninety-two *A. myosuroides* populations studied, 100% exhibited resistance to the recommended field rate of the ACCase herbicide clodinofop-propargyl. 80% of the 46 populations that were untreated with mesosulfuron-methyl + iodosulfuron-methyl-sodium in the year of sampling exhibited resistance to mesosulfuron-methyl + iodosulfuron-methyl-sodium, compared with 96% the 46 populations that were treated with mesosulfuron-methyl + iodosulfuron-methyl-sodium in the year of sampling. When compared with other ALS and ACCase resistance studies of *A. myosuroides* these results are unsurprising, as ALS and ACCase resistance have previously been identified in high frequency in *A. myosuroides* populations sampled from the UK, France, Germany, Belgium, the Netherlands, and Denmark (Chauvel *et al* 2006; Moss *et al* 2007; Délye *et al* 2007; Délye *et al* 2010; Hess *et al* 2012; Hull *et al* 2014; Keshtkar *et al* 2015). Furthermore, the findings of this survey are not unusual when compared with surveys exploring the prevalence of resistance in other weed species and other

herbicide MOA. Among recent surveys alone, ALS resistance has been identified in 98% of Australian *Lolium rigidum* populations tested (Owen *et al* 2014), two studies have identified the frequency of dicolfop-methyl (ACCase) to be present in more than 90% of Australian *L. rigidum* populations sampled (Owen *et al* 2014; Malone *et al* 2013), and 69% of the 144 *Amaranthus rudis* populations sampled from Missouri (USA) were found to exhibit resistance to glyphosate (Rosenbaum and Bradley 2013).

The approach used to conduct the *A. myosuroides* survey in 2011 incorporated some important methodological features that will have affected the results obtained. Firstly, the ninety-two fields surveyed in 2011 were not randomly selected, but were chosen from farms in which resistance to ACCase and ALS modes of action (MOA) had already been confirmed, and from farmers who had expressed that herbicidal control of *A. myosuroides* could be problematic. This is an approach that has been adopted in previous studies (Delye *et al* 2007; Délye *et al* 2010). The use of non-random samples chosen in this way is likely to result in a greater identification of resistance than if a completely random survey had been conducted (e.g. like the random survey conducted by Malone *et al* (2013) on Australian *L. rigidum*) and should be taken into account when considering the extent of herbicide resistance within the region studied.

Second of all, the ninety-two *A. myosuroides* populations sampled in 2011 consisted of 46 pairs of fields, one treated with the ALS inhibitor mesosulfuron-methyl + iodosulfuron-methyl-sodium in the year of collection and one untreated mesosulfuron-methyl + iodosulfuron-methyl-sodium. This led to the finding that

populations treated with mesosulfuron-methyl + iodosulfuron-methyl-sodium in the year of sampling (96% of populations had ALS resistance) exhibited higher levels of ALS resistance when compared to those that were untreated (80% of populations had ALS resistance). Herbicide treatment in the year of seed collection removes the majority of emerged susceptible individuals from the population, leading to an overestimate of resistance frequency at the population level as estimates of resistance frequency are based on seeds produced by surviving (and therefore predominantly resistant) individuals. Therefore, sampling populations that are not treated with herbicide of interest (e.g. ALS or ACCase) in the year of seed collection is able to give a more accurate prediction of the frequency of ALS resistance within the entire population. Previous resistance studies have not taken this into account and may have affected the results obtained, (Chauvel *et al* 2006; Moss *et al* 2007; Délye *et al* 2007; Délye *et al* 2010; Hess *et al* 2012; Malone *et al* 2013; Rosenbaum and Bradley 2013; Hull *et al* 2014; Owen *et al* 2014; Keshtkar *et al* 2015); but it should be taken into account in future herbicide resistance surveys.

Finally, certain considerations have to be taken into account when using the results of any resistance survey, as they need to be placed in an agronomic context, i.e. information about the number of plants surviving herbicide application is required, which again, previous studies have not always taken into consideration (Délye *et al* 2007; Délye *et al* 2010), although some have (Hess *et al* 2012). If the number of individuals surviving herbicide application was small, then the resistance problem may not be great at the time of collection. If there were a large number of individuals surviving herbicide treatment, there will be a large return of resistant seed. From a management perspective therefore, high levels of resistance in seed collected are

indicative of an actual or impending resistance problem, and appropriate IWM approaches should be taken early to reduce the risk of an agronomic problem arising.

7.2 Alopecurus myosuroides resistance mechanisms

One area in which the survey conducted in 2011 has added to existing knowledge of *A. myosuroides* resistance, and herbicide resistance more generally, is in the identification of the frequencies of ALS and ACCase target-site resistance (TSR), enhanced metabolism (EMR) and both mechanisms of resistance together and the potential importance that EMR in particular may have on the evolution of resistance.

ALS and ACCase TSR was prevalent within the 92 UK *A. myosuroides* populations surveyed in 2011, with 49% and 89% of populations possessing at least one ALS TSR mutation and at least one ACCase TSR mutation, respectively. Other studies describing the frequencies of ALS and ACCase target-site resistance in *A. myosuroides* have identified similar results (Moss *et al* 2007; Délye *et al* 2007; Délye *et al* 2010; Hess *et al* 2012; Moss *et al* 2014). One unexpected and novel result from the survey was a lack of homozygous Pro-197-Thr mutations; with phenotyping and genotyping of plants from controlled crosses confirming that there is a lethality associated with homozygous Pro-197-Thr mutations. This identification of only heterozygous Pro-197-Thr mutations is contradicted by studies identifying homozygous Pro-197-Thr mutations in populations of *Lolium rigidum* (Collavo and Sattin 2014), *Papaver rhoeas* (Délye *et al* 2011) and *Alopecurus myosuroides* (Marshall *et al* 2013), but supported by in a previous study by Marshall and Moss (2008); therefore it may be a unique occurrence in *A. myosuroides.* The simulation model presented in this thesis has shown how lethal homozygous Pro-197-Thr

mutation can limit the proportion of Pro-197 target-site resistant individuals within a population, which is important information for designing more effective and sustainable weed management schemes.

Where the novelty lies within the survey conducted in 2011 is the use of high-performance liquid chromatography (HPLC) to quantify the presence and magnitude of ALS and ACCase enhanced metabolism. In previously published studies, enhanced metabolism has been inferred within a phenotypically resistant individual if no TSR mutation was found (Délye *et al* 2007; Moss *et al* 2014). As a result, the occurrences of plants with both TSR and EMR have not been taken into account, potentially underestimating the prevalence of ALS EMR (20% of 570 plants (Moss *et al* 2014)) and ACCase EMR (75% of all plants (Délye *et al* 2007)) identified. Here, with HPLC analysis quantifying ALS and ACCase EMR in 65% and 100% of the 92 populations and 37% and 87% of the 736 plants studied respectively, EMR is undoubtedly an important mechanism in the evolution of resistance.

In herbicide resistance evolution theory, it has been suggested that high frequencies of low-level enhanced metabolism exist within a population's standing genetic variation that enable individuals to survive selection (Neve *et al* 2014). Recurrent selection of these individuals facilitates population growth, increasing the likelihood of rare target-site mutations occurring and being selected (Neve et al 2014). Results presented here, the first of their kind for *A. myosuroides*, show that 39% and 89% of the 92 UK populations surveyed in 2011 exhibit both TSR and EMR to ALS and ACCase MOA, respectively. Through both mechanisms of resistance being present at the population in such high frequencies, this hypothesis appears to be an entirely

plausible process in the evolution of herbicide resistant *Alopecurus myosuroides*: although further conformational studies are needed (e.g. surveys and long term selection experiments).

One other novel experimental finding of this thesis, that pre-existing ACCase enhanced metabolism confers a degree of "pre-selected" enhanced metabolic resistance to ALS modes of action, is important in understanding the processes that underlie the evolution of enhanced metabolism. Although the nature of the genes that confer but ACCase and "pre-adapted" ALS enhanced metabolism can only be speculated – they may be regulatory similar to the *AmGSTF1* identified by Cummins *et al* (2013) - identifying that ACCase EMR resistant *A. myosuroides* populations possess some degree of "pre-adapted" ALS enhanced metabolism puts pressure on existing chemical management regimes. Increased rates of ALS resistance evolution in *A. myosuroides* will lead to a quicker decrease in ALS herbicide efficacy, and with a lack of new MOA and existing MOA being removed from the commercial market, (Lutman *et al* 2013), a greater emphasis will be put on integrated weed management (IWM).

7.3 Resistance trends observed between 2012 and 2014

Seventeen UK populations from the survey conducted in 2011 were re-sampled in 2012, 2013, and 2014. This allowed for a relatively novel approach to studying how the frequencies of ALS and ACCase herbicide resistance, and the mechanisms that endow resistance changed between 2012 and 2014. Across all seventeen populations, the ALS resistance index decreased between 2012 and 2013 before increasing in 2014. The level of ALS TSR in the majority of populations decreased or remained

the same between 2012 and 2013, before increasing in frequency between 2013 and 2014. Conversely, the average amount of ALS herbicide (mesosulfuron) metabolized significantly decreased. These results suggest that target-site resistance might confer increases in ALS resistance within these populations more than enhanced metabolism. For ALS resistance, these results are an indication that early selection within a population may be for enhanced metabolism, as suggested by Neve *et al* (2014) - potentially based on pre-adaptive enhanced metabolism following ACCase selection (as demonstrated in chapter 6) - but that the presence of TSR may decrease the level of ALS enhanced metabolism as TSR provides a higher level of resistance. The ACCase resistance index increased between 2012 and 2014, whereas the frequency of ACCase target-site resistance increased between 2012 and 2013, before decreasing again in 2014 to a level similar to that of 2012. The average amount of ACCase herbicide (fenoxaprop) metabolized increased year on year. These results suggest that the selection for ACCase resistance is still ongoing, but only in those crops where ACCase herbicides are still used (e.g. Oilseed rape).

7.4 Effect of management on resistance evolution

In light of the evolution of *A. myosuroides* resistance to essential ALS and ACCase herbicide MOA in the UK, it is important that *A. myosuroides* management no longer relies solely upon chemical control measures, but that an integrated weed management (IWM) approach is developed to slow the evolution of resistance to ALS and ACCase herbicides.

Using an epidemiological approach, this thesis has alluded to certain factors that increase the rate of ALS and ACCase resistance evolution in *A. myosuroides*. The

greatest levels of ALS and ACCase resistance were identified in populations in which practices associated with ALS and ACCase herbicide application were most frequently applied, e.g. planting of winter wheat is often associated with the application of an ALS herbicide. This ties with a previous study by Evans *et al* (2015) identifying the greatest frequencies of glyphosate resistance in *Amaranthus tuberculatus* in the USA, in populations treated with the greatest frequencies of glyphosate, and agrees with models that state that a more homogeneous management strategies (relying on continuous cropping systems and a single herbicide MOA) will increase the evolution of resistance (Richter 2002).

More generally, the results of this epidemiological study also highlight the importance of using epidemiological approach in identifying the causes and processes involved in the evolution of herbicide resistance. The finding that increased planting of spring crops significantly reduces ALS and ACCase resistance, agrees with previous studies identifying that spring cropping, reduces *A. myosuroides* infestations in the field (Lutman *et al* 2013), and is important information in developing sustainable IWM approaches to herbicide resistance management. Through the findings of this study, and more long-term epidemiological studies such as this, a greater understanding the temporal evolution of *A. myosuroides* herbicide resistance in relation to management can be distinguished, so that more effective *A. myosuroides* resistance control strategies can be developed.

7.5 Herbicide resistance modelling

Simulation models, parameterised and validated by small accompanying experiments, are a vitally important tool in identifying and understanding the

biological, evolutionary, and ecological components that drive selection for herbicide resistance (Renton *et al* 2014). Therefore, one aim of this thesis was to develop a model of *A. myosuroides* herbicide resistance evolution capable of relating changes in the frequency of resistance and resistance mechanisms to field management factors.

Ultimately, it was found that the patterns and changes in ALS and ACCase resistance evolution predicted by the model were unable to accurately correlate resistance data collected from the field. A number of published herbicide resistance models tackling a range of questions related to the evolution of herbicide resistance in a number of agricultural weed species have been developed (Gardner *et al* 1998; Diggle *et al* 2003; Neve *et al* 2003a, 2003b; Jacquemin *et al* 2009; Neve 2008; Neve *et al* 2010; Renton *et al* 2011; Richter *et al* 2002 Richter *et al* 2012; Richter *et al* 2014; Bagavathiannan *et al* 2014). However, none of these models have been validated using field data. Therefore, the model presented in this thesis is still a significant step in the development of a model capable of addressing questions regarding the evolution of ALS and ACCase resistance in *A. myosuroides,* as it has allowed factors and parameters within the model that may be incorrect to be identified (as discussed in section 7.8.4).

7.6 Conclusions

- Populations untreated with the ALS herbicide mesosulfuron-methyl + iodosulfuron-methyl-sodium in the year of sampling exhibit lower levels ALS resistance, as they represent the whole population, not only resistant individuals surviving herbicide application. ALS untreated populations

therefore offer a more accurate representation of the frequency of ALS resistance.

- ACCase and ALS resistance is widespread in the UK, being identified in 100% (of 92 populations) and 80% (of 46 populations untreated with mesosulfuron-methyl + iodosulfuron-methyl-sodium in 2011) respectively.
- With ALS and ACCase EMR identified in 65% and 100% of the 92 populations studied, and ALS and ACCase TSR identified in 38% and 89% of populations respectively, both mechanisms of resistance are prevalent within the UK. Importantly, 39% and 89% of the 92 UK populations surveyed in 2011 exhibit both TSR and EMR to ALS and ACCase MOA respectively.
- Across the seventeen populations studied between 2012 and 2014, ALS resistance index decreased between 2012 and 2013 before increasing in 2014; the level of ALS TSR also exhibited this pattern. Conversely, the average amount of ALS herbicide (mesosulfuron) metabolized decreased.
- Across the seventeen populations studied between 2012 and 2014, the ACCase resistance index increased between 2012 and 2014, whereas the frequency of ACCase target-site resistance increased between 2012 and 2013, before decreasing again in 2014. The average amount of ACCase herbicide (fenoxaprop) metabolized increased year on year.
- The identification of high levels of ALS resistance within a sampling year (resulting primarily from the mechanism of TSR), are associated with management practices related to the frequent planting winter wheat (e.g. early sowing date and ALS herbicide application).

- Increased planting of spring crops significantly reduces the level of ALS and ACCase phenotypic resistance.
- A simulation model was developed to describe *A. myosuroides* herbicide resistance evolution, however the model could not be validated when parameterized with resistance and management data collected from the UK.
- Within the 2011 survey, homozygous Pro-197-Thr ALS TSR mutations were absent. Phenotyping and genotyping of plants from controlled crosses confirmed the lethality associated with homozygous Pro-197-Thr mutations.
- Populations that possessed pre-existing ACCase EMR exhibited significant increases in ALS resistance after two years of selection with an ALS herbicide, whereas a population without ACCase EMR did not.

7.7 Suggested Future Work

7.7.1 Epidemiological studies of resistance evolution

To advance the epidemiological study into the evolution of herbicide resistance in *Alopecurus myosuroides* presented here, a greater sample size needs to be studied. Here, 17 populations from the UK were sampled between 2012 and 2014. A larger sample size would enable greater statistical power in identifying significant relationships between resistance and historical weed management data. Additional data (similar to that included in a study by Evans *et al* (2015)) regarding soil, weather and the distribution of *A. myosuroides* in the field should also be collected in any future epidemiological study, to investigate whether any correlation between the frequency of resistance measures can be identified with these additional factors. The collection of additional field data will also be important information to aid the

development and validation of a simulation model of *A. myosuroides* herbicide resistance evolution.

7.7.2 Pro-197-Thr segregation and fitness studies

To identify whether the absence of homozygous Pro-197-Thr mutations are a pre-zygotic or post-zygotic effect, further work is needed in the form of the genotyping of un-germinated seed. If individuals possessing homozygous Pro-197-Thr mutations are present in the un-germinated seed, this is an indication that seeds with the mutations are present but do not germinate (a post-zygotic lethal effect); If individuals possessing homozygous Pro-197-Thr mutations are not present in the un-germinated seed, this is an indication that seeds with the mutations do not mature and are therefore not present (a pre-zygotic lethal effect). Additionally, the underlying physiology of the lethality needs to be investigated further. Identifying the activity of susceptible, heterozygous, and (if possible) homozygous ALS enzymes in *A. myosuroides*, as Li *et al* (2012) has done for *Raphanus raphanistrum,* will indicate if there is an increase or decrease in the activity of homozygous Pro-197-Thr mutations that may contribute to the observed lethality.

7.7.3 Selection of ALS enhanced metabolism from a background of ACCase enhanced metabolism

To further the investigation the link between the selection of ALS and ACCase enhanced metabolism, the levels of ALS and ACCase enhanced metabolism present within individual plants could be quantified using high-performance liquid chromatography (HPLC) before selection. A number of discrete groups could then be created (e.g. low ALS EMR, high ALS EMR, high ACCase EMR, etc.) and the bulk crossing of groups could then be based on HPLC results rather than survival of

herbicide selection. This will enable further quantification of the effect of pre-existing ACCase enhanced metabolism on the selection of ALS enhanced metabolism, e.g. do lower levels of ACCase enhanced metabolism mean a slower ALS enhanced metabolism selection rate? This approach could be extended to investigate whether pre-existing ALS enhanced metabolism results in an increase in the rate of selection of ACCase enhanced metabolism. Both of the suggested experiments will expand current knowledge regarding the number of genes involved in enhanced metabolic resistance (Petit *et al* 2010; Rosenhauer *et al* 2015).

7.7.4 Modelling the evolution of herbicide resistance in A. myosuroides

To further the work of this thesis in the creation and validation of a simulation model of *A. myosuroides* herbicide resistance evolution, the issues identified with modelling the mechanisms of resistance represented in the model - particularly enhanced metabolism (section 4.5.5.1) - need to be addressed. At present, single gene endows some level of enhanced metabolism to both ALS and ACCase modes of action within the model. With the exact genetics of enhanced metabolism (a quantitative trait) unknown, it was therefore not unreasonable to represent enhanced metabolism as a single gene trait for both modes of action. A number of approaches have already been tried when modelling a quantitative resistance trait such as enhanced metabolism: Renton *et al* (2011) modelled enhanced metabolism as a polygenic trait involving several genes, while Gardner *et al* (1998) modelled enhanced metabolism as quantitative selection from a normal distribution. Either of these approaches could be adopted and incorporated into the model of *A. myosuroides* herbicide resistance evolution to try and improve the representation of ALS and ACCase enhanced metabolism.

The most productive step forward in improving the model however, would be to incorporate recent information gained from studies of enhanced metabolism by the likes of Rosenhauer *et al* (2015) and Cummins *et al* (2013). Rosenhauer *et al* (2015) estimated the minimum number of genes involved in enhanced metabolism for a number of MOA (i.e. a minimum of two genes for ALS enhanced metabolism), Cummins *et al* (2013) identified the regulatory gene *AmGSTF1,* and Gaines *et al* (2014) identified several genes associated with the development of EMR. By adapting the current three-gene model so that it represents only one MOA (e.g. ALS) - with one gene representing TSR and the other two genes representing enhanced metabolism - by assuming that one of EMR genes is regulatory (similar to the gene identified by Cummins *et al* (2013)), the recent experimental findings by can Rosenhauer *et al* (2015) and Cummins *et al* (2013) be tested using the model. If validated by data from the field, this will be an important step forward in creating a model capable of accurately modelling *A. myosuroides* herbicide resistance evolution, this might help to select the most appropriate Integrated Weed Management strategy adapted to each field.

Although a number of herbicide resistance models have been published for a number of species (Richter *et al* 2002; Diggle *et al* 2003; Neve *et al* 2003a, 2003b; Neve 2008; Jacquemin *et al* 2009; Neve *et al* 2010; Richter *et al* 2012; Bagavathiannan *et al* 2014), this is one of the first models (and the first for *A. myosuroides*) to attempt to validate the model using field data. The parameter estimates used within the model (estimated from published literature) and the quality of the field data used to validate it, are factors that would also have had an impact on the modelling output. To fully understand the affect of these on the model, a greater range of each parameter

estimates would have to be tried within the model, and the model would have to be compared to a greater number of field data studies.

8.0 References

Bagavathiannan, M. V., Norsworthy, J. K., Smith, K. L., and Neve, P. (2014). Modeling the Simultaneous Evolution of Resistance to ALS- and ACCase-Inhibiting Herbicides in Barnyardgrass (*Echinochloa crus-galli*) in Clearfield® Rice. *Weed Technology*. **28**: 89-103.

Baker, H. G. (1974). The Evolution of Weeds. *Annual Review of Ecology and Systematics*. **5**: 1 – 24.

Bayer CropScience. (2012). Bayer Expert Guide – Blackgrass management in winter cereals. Cambridgeshire: BayerCrop Science Ltd.

Bridges, D. C., Anderson, R. L. (1992). Crop Losses Due to Weeds in the United States. Weed Science Society of America. Champaign, Illinois.

Burt, A., Trivers, R. (2006). Genes in Conflict. The Belknap Press of Harvard University Press: Cambridge, Massachusetts/London, England.

Busi, R., and Powles, S.B. (2009). Evolution of glyphosate resistance in a Lolium rigidum population by glyphosate selection at sublethal doses. *Heredity*. **103**: 318-325.

Busi, R., Gaines, T. A., Walsh, M. J., and Powles, S. B. (2012), Understanding the potential for resistance evolution to the new herbicide pyroxasulfone: field selection at high doses versus recurrent selection at low doses. *Weed Research*. **52**: 489–499.

Busi, R., Neve, P., and Powles, S. B. (2013) Evolved polygenic herbicide resistance in Lolium rigidum by low-dose herbicide selection within standing genetic variation. *Evolutionary Applications*. **6:** 231-242.

Campbell, N. A., Reece, J. B. (2005). Biology. 7th edn. Pearson Education Inc: San Francisco.

Cavan, G., Biss, P., Moss, S. R. (1998). Localized Origins of Herbicide Resistance in *Alopecurus Myosuroides*. *Weed Research*. **38**: 239–245.

Chauvel, B., Guillemin, J. P., Dessaint, F., and Délye, C. (2006). Regional study of herbicide resistance of *Alopecurus myosuroides* Huds. in France. *Journal of Plant Disease and Protection*. [online] http://www.jpdp-online.com/Content/Special-Issue-XX/Chapter-2/Regional-study-of-herbicide-resistance-of-i-Alopecurus-myosuroides-i-Huds-in-France,QUlEPTEwMjM3NSZNSUQ9NzI3ODc.html. (Accessed 20/8/2015)

Cocker, K.M., Moss, S. R., Coleman, J. O. D. (1999). Multiple mechanisms of resistance to fenoxaprop-P-ethyl in United Kingdom and other European populations of herbicide-resistant *Alopecurus myosuroides* (Black-Grass). *Pesticide Biochemistry and Physiology*. **65**:169-180

Colbach, N., and Sache, I. (2001). Blackgrass (Alopecurus myosuroides Huds.) seed dispersal from a single plant and its consequences on weed infestation. *Ecological Modelling*. **139**: 201–219.

Colbach, N., D¨urr, C., Roger-Estrade, J., Chauvel, B., and Caneill, J. (2006a). AlomySys: Modelling black-grass (Alopecurus myosuroides Huds.) germination and emergence, in interaction with seed characteristics, tillage and soil climate I. Construction. *Europ. J. Agronomy*. **24**: 95–112.

Colbach, N., Busset, H., Yamadaa, O., D¨urr, C., and Caneill, J. (2006b). AlomySys: Modelling black-grass (Alopecurus myosuroides Huds.) germination and emergence, in interaction with seed characteristics, tillage and soil climate II. Evaluation. *Europ. J. Agronomy*. **24**: 113–128

Collavo, A., and Sattin, M. (2014). First glyphosate-resistant *Lolium* spp. biotypes found in a European annual arable cropping systems also affected by ACCase and ALS resistance. *Weed Research*. **54**: 325–334.

Coulson, T., Catchpole, E. A., Albon, S. D., Morgan, B. J., Pemberton, J. M., Clutton-Brock, T. H., Crawley, M. J., and Grenfell, B. T. (2001). Age, sex, density, winter weather, and population crashes in Soay sheep. *Science,* **292**: 1528-1531.

Cousens, R. and Moss, S. R. (1990), A model of the effects of cultivation on the vertical distribution of weed seeds within the soil. *Weed Research*. **30**: 61–70.

Cummins, I., Wortley, D. J., Sabbadin, F., He, Z., Coxon, C. R., Straker, H. E., Edwards, R. (2013). Key role for a glutathione transferase in multiple-herbicide resistance in grass weeds. *PNAS.* **110**: 5812–5817.

Cussans, G. W. (1991). Weed Control Strategies – Research Progress (Review). 2.1-2.19.

DEFRA. (2013). Pesticide Usage Survey Report 250: Arable Crops in the United Kingdom 2012. United Kingdom, York: DEFRA [online] *Available from: https://secure.fera.defra.gov.uk/pusstats/surveys/documents/arable2012.pdf* (Accessed: 25th July 2015).

Délye, C. (2005). Weed resistance to acetyl coenzyme A carboxylase inhibitors: an update. *Weed Science.* **53**: 728–746.

Délye, C. (2012). Unravelling the genetic bases of non-target-site-based resistance (NTSR) to herbicides: a major challenge for weed science in the forthcoming decade. *Pest Manag Sci.* **69**:176-87.

Délye, C., Menchari, Y., Guillemin, J. P., Matéjicek, A., Michel, S., Camilleri, C. and Chauvel, B. (2007). Status of black grass (*Alopecurus myosuroides*) resistance to acetyl-coenzyme A carboxylase inhibitors in France. *Weed Research.* **47**: 95–105.

Délye, C., Michel, S., Bérard, A., Chauvel, B., Brunel, D., Guillemin, J. P., Dessaint, F., and Le Corre, V. (2010). Geographical variation in resistance to acetyl-coenzyme A carboxylase-inhibiting herbicides across the range of the arable weed Alopecurus myosuroides (black-grass). *New Phytol.* **186**: 1005-17.

Délye, C., Pernina, F., Scarabel, L. (2011a). Evolution and diversity of the mechanisms endowing resistance to herbicides inhibiting acetolactate-synthase (ALS) in corn poppy (*Papaver rhoeas* L.). *Plant Science.* **180**: 333–342.

Délye, C., Gardin, J. A. C., Boucansaud, K., Chauvel, B. and Petit, C. (2011b), Non-target-site-based resistance should be the centre of attention for herbicide resistance research: *Alopecurus myosuroides* as an illustration. *Weed Research.* **51:** 433–437.

Délye, C., Jasieniuk, M., and Le Corre, V. (2013) Deciphering the evolution of herbicide resistance in weeds. Trends in Genetics. **29**: 649 – 658.

Diggle, A. J., Neve, P. B. and Smith, F. P. (2003). Herbicides used in combination can reduce the probability of herbicide resistance in finite weed populations. *Weed Research*. **43**: 371–382.

Dündar, E. (2004). Physiological and Molecular Analysis of a Valine Resistant Mutant in *Arabidopsis Thaliana. BAÜ Fen Bil. Enst. Dergisi.* **6:** 4-15.

Evans, J. A., Tranel, P. J., Hager, A. G., Schutte, B., Wu, C., Chatham, L. A. and Davis, A. S. (2015). Managing the evolution of herbicide resistance. *Pest. Manag. Sci*. doi: 10.1002/ps.4009

FAO, Food and Agriculture Organization of the United Nations. (2009). FAO's Director-General on How to Feed the World in 2050. *Population and Development Review,* **35**: 837–839.

FAO, Food and Agriculture Organization of the United Nations. (2013a). State of Food Insecurity in the World 2013. Rome, Italy: FAO. [online] *Available from: http://www.fao.org/docrep/018/i3434e/i3434e00.htm* (Accessed: 25th July 2015).

FAO, Food and Agriculture Organization of the United Nations (2013b). FAOSTAT. [online] *Available from: http://faostat.fao.org/site/291/default.aspx* (Accessed July 25, 2015).

Fitter, R., and Fitter, A. (1984) Collins guide to the grasses, sedges, rushes and ferns of Britain and Northern Europe. Collins: London.

Gaines, T. A., Zhang, W., Wang, D., Bukun, B., Chisholm, S. T., Shaner, D. L., Nissen, S. J., Patzoldt, W. L., Tranel, P. J., Culpepper, A. S., Grey, T. L., Webster, T. M., Vencill, W. K., Sammons, R. D., Jiang, J., Preston, C., Leach, J. E., and Westra, P. (2010). Gene amplification confers glyphosate resistance in *Amaranthus palmeri. PNAS*. **107**: 1029–1034.

Gaines, T. A., Lorentz. L., Figge, A., Herrmann, J., Maiwald, F., Ott, M., Han, H., Busi, R., Yu, Q., Powles, S. B., and Beffa, R. (2014). RNA-Seq transcriptome analysis to identify genes involved in metabolism-based diclofop resistance in *Lolium rigidum. The Plant Journal*. **78**: 865–876.

Gardner, S. N., Gressel, J. and Mangel, M. (1998). A revolving dose strategy to delay the evolution of both quantitative vs major monogene resistances to pesticides and drugs. *International Journal of Pest Management.* **44**: 161-180

Geissel, H (2004). Leaving Certificate Agricultural Science. 3rd edn. Germany: CRS publications. [online] *Available from: http://www.crsbooks.net/agscience/weeds.html#black* (Accessed July 25, 2015).

Greenwood, B. M. (1997). The epidemiology of malaria. *Annals of Tropical Medicine and Parasitology.* **91:** 763-769.

Grime, P., Hodgson, J. G., & Hunt, R. (1988). Comparative Plant Ecology. Castlepoint Press: United Kingdom.

Hall, L. M., Holtum, J. A. M., and Powles, S. B. (1994). Mechanisms responsible for cross resistance and multiple resistance. In Herbicide Resistance in Plants: Biology and Biochemistry. Lewis Publishers: Boca Raton, FL.

Hamann, M., Grech, A., Wolanski, E., and Lambrechts, J. (2011). Modelling the fate of marine turtle hatchlings. *Ecological Modelling*. **222**: 1515-1521.

Harper, J. L. (1956). The Evolution of weeds in Relation to Resistance to Herbicides. *In: Proceedings of the 1956 British weed control conference. The British Crop Protection Council: UK.* 179–188.

Hawkley, P. M., and Jones, A. M. (2009). The changing epidemiology of resistance. *Journal of Antimicrobial Chemotherapy.* **64**: Suppl. 1, i3–i10

Heap, I. (1997). The Occurrence of Herbicide-resistant Weeds Worldwide. *Pestic. Sci.* **51**: 235-243.

Heap, I. (2014). Global perspective of herbicide-resistant weeds. Pest Management Science. **70**: 1306–1315.

Heap, I. (2015). The International Survey of Herbicide Resistant Weeds. [online] *Available from: http://weedscience.org* (Accessed September 2, 2015).

Hess, M., Beffa, R., Kaiser, J., Laber, L., Menne, H., & Strek, H. (2012). Status and development of ACCase and ALS inhibitor resistant black-grass (*Alopecurus myosuroides* Huds.) in neighboring fields in Germany. *25th German Conference on Weed Biology and Weed Control, March 13-15, 2012, Braunschweig, Germany.* [online] *Available from:* http://pub.jki.bund.de/index.php/JKA/article/view/1726 (Accessed 26th July 2015).

HGCA. (2010). Managing weeds in arable rotations – A guide, Summer 2010. Warwickshire: HGCA. HGCA Publications: UK.

Hilton, H. W. (1957), Herbicide tolerant strains of weeds. *Hawaiian Sugar Plant Assoc. Ann. Rep.* p 69.

Holm, L. G. (1991). The World's worst wVeeds: distribution and biology. 2nd Edition. East-West Center by the University Press of Hawaii: Honolulu, USA.

Hubbard, C. E. (1984). Grasses: A Guide to Their Structure, Identification, Uses, and Distribution in the British Isles. 3rd Edition. Penguin Books Limited.: Harmondsworth.

Hull, R., Tatnell, L. V., Cook, S. K., Beffa, R., and Moss, S. R. (2014). Current status of herbicide-resistant weeds in the UK. *Aspects of Applied Biology.* **127**.

Jacquemin, B,. Gasquez, J,. and Reboud, X. (2009). Modelling binary mixtures of herbicides in populations resistant to one of the components: evaluation for resistance management. *Pest Manag Sci.* **65**: 113 - 121.

Jasieniuk, M., Brule-Babel, A. L., and Morrison, I. N. (1996). The Evolution and Genetics of Herbicide resistance in weeds. *Weed Science*. **44**. 176 – 193.

Jørgensen, S. E., and Fath, B. D. (2011). Fundamentals of Ecological Modelling: Applications in Environmental Management and Research. 4th Ed. Elsevier: Amsterdam, the Netherlands.

Kaper, J. B., Morris, J. G., Levine, M, M. (1995). Cholera. *Clinical Microbiology Reviews*. **8**: 48 – 56.

Kearney, P.C., Kaufman, D.D. (1975) Herbicides. Vol.1, chemistry, degradation and mode of action. 2nd edn. M. Dekker: New York.

Keshtkar E., Mathiassen, S. K., Moss, S. R., Kudsk, P. (2015). Resistance profile of herbicide-resistant Alopecurus myosuroides (black-grass) populations in Denmark. Crop Protection. **69**: 83-89.

Kilmarx, P. H. (2009). Global epidemiology of HIV. *Current opinion in HIV and AIDS.* **4:** 240-246.

Koella, J. C., Lynch, P. A., Thomas, M. B., and Read, A. F. (2009). Towards

evolution-proof malaria control with insecticides. *Evolutionary applications*. **2**: 469 – 480.

Kozielska, M., F J Weissing, F. J., Beukeboom, L. W., and Pen, I. (2010). Segregation distortion and the evolution of sex-determining mechanisms. *Heredity.* **104:** 100–112.

Lagator, M., Vogwill. T, Colegrave, N., and Neve, P. (2013a). Herbicide cycling has diverse effects on evolution of resistance in *Chlamydomonas reinhardtii*. *Evol Applic*. **6:**197–206.

Lagator, M., Vogwill, T., Mead, A., Colegrave, N., and Neve, P. (2013). Herbicide mixtures at high doses slow the evolution of resistance in experimentally evolving populations of *Chlamydomonas reinhardtii*. *New Phytol*. **198:** 938–945.

Langemann ,D., Richter, O., and Vollrath, A. (2013). Multi-gene-loci inheritance in resistance modeling. *Mathematical Biosciences*. **242**: 17-24.

Last, J. M. (2001). Dictionary of epidemiology. 4th ed. Oxford University Press: New York.

Li, M., Yu, Q., Han, H., Vila-Aiub, M. and Powles, S. B. (2013), ALS herbicide resistance mutations in Raphanus raphanistrum: evaluation of pleiotropic effects on vegetative growth and ALS activity. *Pest. Manag. Sci.* **69**: 689–695.

Lutman, P. J. W., Moss, S. R., Cook, S. & Welham, S. J. (2013). A review of the effects of crop agronomy on the management of *Alopecurus myosuroides*. *Weed Research*. **53:** 299–310.

Malone, J. M., Boutsalis, P., Baker, J. & Preston, C. (2013). Distribution of herbicide-resistant acetyl-coenzyme A carboxylase alleles in *Lolium rigidum* across grain cropping areas of South Australia. *Weed Research* **54,** 78–86.

Manalil, S., Busi, R., Renton, M., and Powles, S.B. (2011). Rapid Evolution of Herbicide Resistance by Low Herbicide Dosages. *Weed Science*. **59**: 210–217

Marshall, R., and Moss, S. R. (2008). Characterisation and molecular basis of ALS inhibitor resistance in the grass weed *Alopecurus myosuroides*. *Weed Research*. **48**: 439–447.

Marshall, R., Hanley, S. J., Hull, R., and Moss, S. R. (2013), The presence of two different target-site resistance mechanisms in individual plants of *Alopecurus myosuroides* Huds., identified using a quick molecular test for the characterisation of six ALS and seven ACCase SNPs. *Pest. Manag. Sci.* **69**: 727–737.

Mayhew, P. (2006). Discovering Evolutionary Ecology: Bringing together ecology and evolution. Oxford University Press: New York.

Menchari, Y., Camilleri, C., Michel, S., Brunel, D., Dessaint, F., Le Corre, V., and Délye, C. (2006). Weed response to herbicides: regional-scale distribution of herbicide resistance alleles in the grass weed *Alopecurus Myosuroides. New Phytologist.* **171**: 861–874.

Menchari, Y., Chauvel, B., Darmency, H. and Délye, C. (2008), Fitness costs associated with three mutant acetyl-coenzyme A carboxylase alleles endowing herbicide resistance in black-grass *Alopecurus myosuroides*. *Journal of Applied Ecology*, **45**: 939–947

Mithila, J., Hall, C., Johnson, W.G., Kelley, K, B., and Riechers, D. E. (2011). Evolution of Resistance to Auxinic Herbicides: Historical Perspectives, Mechanisms of Resistance, and Implications for Broadleaf Weed Management in Agronomic Crops. *Weed Science*. **59**: 445-457.

Moss, S. R. (1983). The production and shedding of Alopecurus myosuroides Huds. seeds in winter cereals crops. *Weed Research*. **23**: 45-51

Moss, S. R. (1985). The survival of Alopecurus myosuroides Huds. seeds in soil. *Weed Research*. **25**: 201-211.

Moss, S. R. (2013). Black-grass (*Alopecurus myosuroides*) Revised edition. United Kingdom: Rothamsted Research. [online] *Available from: http://www.rothamsted.ac.uk/sites/default/files/attachments/2014-01-09/2%20Black-grass%20leaflet%20revised%20easy%20read%20format%2028May13.pdf* (Accessed July 25, 2015).

Moss, S. R., and Cussans, G. W. (1985). Variability in the susceptibility of *Alopecurus myosuroides* (Black-grass) to chlorotoluron and isoproturon. *Aspects of Applied Biology.* **9**: 91-98.

Moss, S. R., Perryman, S. A. M., and Tatnell, L. V. (2007). Managing Herbicide-

Resistant Blackgrass (Alopecurus myosuroides): Theory and Practice. *Weed Technology*. **21**: 300-309.

Moss, S. R., Hull, R. I., Marshall, R., Perryman, S. A. M. (2014). Changes in the incidence of herbicide-resistant *Alopecurus myosuroides* (black-grass) in England, based on sampling the same random fields on two occasions. *Aspects of Applied Biology* (*Crop Production in Southern Britain, Precision decisions for profitable cropping)*. **127:** 39–48.

Naylor, R. E. L. (1972). *Alopecurus Myosuroides* Huds. (A. Agrestis L.). *Journal of Ecology*. **60**: 611-622

Naylor, R. E. L. & Drummond, C. (2002) Weed Management Handbook. 9th ed^{n}. Blackwell Science Ltd: Oxford.

Neve, P. (2007). Challenges for herbicide resistance evolution and management: 50 years after Harper. *Weed Research.* **47**: 365–369.

Neve, P. (2008). Simulation modelling to understand the evolution and management of glyphosate resistant in weeds. *Pest Management Science.* **64**: 392-401.

Neve, P., Diggle, A. J., Smith, F. P. and Powles, S. B. (2003), Simulating evolution of glyphosate resistance in *Lolium rigidum* I: population biology of a rare resistance trait. *Weed Research,* **43**: 404–417.

Neve, P., Diggle, A. J., Smith, F. P. and Powles, S. B. (2003), Simulating evolution of glyphosate resistance in *Lolium rigidum* II: past, present and future glyphosate use in Australian cropping. *Weed Research*. **43**: 418–427.

Neve, P., and Powles, S. (2005). High survival frequencies at low herbicide use rates in populations of Lolium rigidum result in rapid evolution of herbicide resistance. *Heredity.* **95**: 485–492.

Neve, P., Norsworthy, J. K., Smith, K. L. and Zelaya, I. A. (2010). Modelling evolution and management of glyphosate resistance in *Amaranthus palmeri*. *Weed Research,* **51**: 99–112.

Neve, P., Busi, R., Renton, M. and Vila-Aiub, M. M. (2014), Expanding the eco-evolutionary context of herbicide resistance research. *Pest. Manag. Sci.* **70**: 1385–1393.

OED. (2015). "weed, n.1". Oxford University Press. [online] *Available from: http://www.oed.com/view/Entry/226761* (accessed July 25, 2015).

Oerke, C. E. (2006). Crop Losses to Pests. *Journal of Agricultural Science.* **144**: 31–43.

Online Atlas of British and Irish Flora. (2013) [online] *Available from: http://www.brc.ac.uk/plantatlas/* (Accessed March 2013)

Otto, S. P., and Day, T. (2007). A biologist's guide to mathematical modeling in ecology and evolution. Princeton University Press: New Jersey, USA.

Owen, M.J., Martinez, N.J., and Powles, S. B. (2014). Multiple herbicide-resistant *Lolium rigidum* (annual ryegrass) now dominates across the Western Australian grain belt. *Weed Research*. **54**: 314 – 324.

Peterson, D. (2010). Herbicide Mode of Action. Kansas State University [online] *Available from: https://store.extension.iastate.edu/Product/Herbicide-Mode-of-Action. (Accessed* July 25, 2015).

Petersen, J., Dresbach-Runkel, M. and Wagner, J. (2011). A method to determine the pollen-mediated spread of target-site resistance to acetylcoenzyme A carboxylase inhibitors in black grass (*Alopecurus myosuroides* Huds.). *Journal of Plant Diseases and Protection*. **117**: 122–128.

Petit, C., Bay, G., Pernin, F., Délye, C. (2010). Prevalence of cross- or multiple resistance to the acetyl-coenzyme A carboxylase inhibitors fenoxaprop, clodinafop and pinoxaden in black-grass (Alopecurus myosuroides Huds.) in France. *Pest Manag Sci.* **66**: 168-77.

Pfaller, M. A. (2012). Antifungal Drug Resistance: Mechanisms, Epidemiology, and Consequences for Treatment. *The American Journal of Medicine.* **125**: S3-213.

Phillipson, A. (1974). Survey of the presence of wild oat and blackgrass in parts of the United Kingdom. *Weed Research*. **14**: 123-135.

Powles, S. B., Yu, Q. (2010). Evolution in Action: Plants Resistant to Herbicide. *Annu. Rev. Plant Biol.* **61**: 317–47.

R Core Development Team (2012). R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria.

Raviglione, M. C., Snider, D. E., Kochi, A. (1995). Global epidemiology of tuberculosis - morbidity and mortality of a worldwide epidemic**.** *Jama-journal of the American medical association***. 273**: 220-226

Renton, M., Diggle, A., Manalil, S., and Powles, S. (2011). Does cutting herbicide rates threaten the sustainability of weed management in cropping systems? *Journal of theoretical biology*. **283**: 14-27.

Renton, M., Busi, R., Neve, P., Thornby, D. and Vila-Aiub, M. (2014), Herbicide resistance modelling: past, present and future. *Pest. Manag. Sci*. **70**: 1394–1404.

Richter, O., Zwerger, P. and Böttcher, U. (2002), Modelling spatio-temporal dynamics of herbicide resistance. *Weed Research*, **42**: 52–64.

Richter, O., Langemann, D., and Rummland, J. (2012). A discrete evolutionary genetic model for the emergence of metabolic resistance. *25th German Conference on Weed Biology and Weed Control*. March 13-15, 2012 Braunschweig, Germany.

Roebuck, J. F. (1987). Agricultural problems of weeds on the crop headland. B.C.P.C. Monograph (35).

Rosenbaum, K. K., and Bradley, K. W. (2013). A Survey of Glyphosate-Resistant Waterhemp (*Amaranthus rudis*) in Missouri Soybean Fields and Prediction of Glyphosate Resistance in Future Waterhemp Populations Based on In-Field Observations and Management Practices. *Weed Technology*. **27**: 656-663.

Rosenhauer, M., Felsenstein, F. G., Piepho, H. P., Höfer, M., Petersen, J. (2015). Segregation of non-target-site-based resistance to herbicides in multiple-resistant *Alopecurus myosuroides* plants. *Weed Research*. **55**: 298–308.

Roux, F., Gasquez, J., and Reboud, X. (2004). The Dominance of the Herbicide Resistance Cost in Several Arabidopsis thaliana Mutant Lines. *Genetics*, **166**: 449 – 460.

Russell, S., Barron, A. B., and Harris, D. (2013). Dynamic modelling of honey bee

(*Apis mellifera*) colony growth and failure. *Ecological modelling*. **265**: 158 – 169.

Ryan, G. F. (1970) Resistance of common groundsel to simazine and atrazine. *Weed Science.* **18**: 614-16.

Seefeldt, S. S., Jensen, J. E. & Fuerst, E. P. (1995) Log-logistic analysis of herbicide dose response relationships. *Weed Technology*. **9**: 218-227.

Sinden, J., Jones, R., Hester, S., Odom, D., Kalisch, C., James, R., and Cacho, J. (2004). The economic impact of weeds in Australia. Adelaide, Australia. [Online] *Available from:* https://www.cbd.int/financial/values/australia-economicweeds.pdf. (Accessed: 27th December 2015)

Stark, G. (2011). EU Pesticide Legislation – An Update. *Aspects of Applied Biology.* **106**: 259 – 262.

Streibig, J. C. (2003). Assessment of herbicide effects. [Online] Available from: http://www.ewrs.org/et/docs/herbicide_interaction.pdf. (Accessed 28th December 2015)

Swain, A. J., Hughes, Z. S., Cook, S. K. and Moss, S. R. (2006). Quantifying the dormancy of *Alopecurus myosuroides* seeds produced by plants exposed to different soil moisture and temperature regimes. *Weed Research*. **46**: 470–479.

Tranel, P., and Wright, T. (2002). Resistance of weeds to ALS-inhibiting herbicides: what have we learnt? *Weed Science*. **50**. 700 – 712.

UK Government. (2013). A Strategy for Agricultural Technologies. London, United Kingdom [online] *Available from:* https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/227259/9643-BIS-UK_Agri_Tech_Strategy_Accessible.pdf (Accessed: 25th July 2015).

United Nations. (2004). The United Nations on World Population in 2300. *Population and Development Review*, **30**: 181–187.

United Nations. (2013). World Population Prospects: The 2012 Revision. New York, USA. [Online] *Available from:* http://esa.un.org/unpd/wpp/publications/Files/WPP2012_HIGHLIGHTS.pdf. (Accessed: 27th December 2015)

Vila-aiub, M. M., Neve, P., Steadman, K. J. and Powles, S. B. (2005), Ecological fitness of a multiple herbicide-resistant *Lolium rigidum* population: dynamics of seed germination and seedling emergence of resistant and susceptible phenotypes. *Journal of Applied Ecology*, **42**: 288–298.

Vila-Aiub, M. M., Neve, P and Powles, S. B. (2009). *Fitness costs associated with evolved herbicide resistance alleles in plants.* New Phytologist, **184**: 751-767.

Vila-Aiub, M. M., Neve, P., Roux, F. (2011). A unified approach to the estimation and interpretation of resistance costs in plants. *Heredit*y. **107**: 386-94.

Weed Technology. (1998). Technical Notes. *Weed Technology*. **12**. 789.

Weir, B. S., and Cockerham, C. C. (1984). Estimating F-Statistics for the Analysis of Population Structure. *Evolution*. **38**: 1358-1370.

Yu, Q., Han, H., Li, M., Purba, E., Walsh, M. J., and Powles, S. B. (2012), Resistance evaluation for herbicide resistance–endowing acetolactate synthase (ALS) gene mutations using *Raphanus raphanistrum* populations homozygous for specific ALS mutations. *Weed Research.* **52**: 178–186.

Yu, Q., and Powles, S. B. (2014). Resistance to AHAS inhibitor herbicides: current understanding. *Pest Manag Sci.* **70**:1340-50.

Zhan, J., Fitt, B. D. L., Pinnschmidt, H. O., Oxley, S. J. P., and Newton, A. C. (2008). Resistance, epidemiology and sustainable management of *Rhynchosporium secalis* populations on barley. *Plant Pathology*. **57**: 1–14.

9.0 Abbreviation list

ACCase	Acetyl Co enzyme A synthase
ALS	Acetolactate synthase
AOPP	Aryloxyphenoxypropionate
Atlantis	ALS herbicide mesosulfuron-methyl + iodosulfuron-methyl-sodium
CHD	Cyclohexanedione
ED	Estimated dose
EMR	Enhanced metabolism
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
GLM	Generalized linear model
H_3PO_4	Phosphoric acid
HPLC	High performance liquid chromatography
IWM	Integrated weed management
Min til	Minimum tillage
MOA	Mode of action
NTSR	Non target-site resistance
PCR	Polymerase chain reaction
SOA	Site of action
SNP	Single nucleotide polymorphism
TSR	Target-site resistance